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Stomach content analysis in freshwater fish feeding ecology

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Preface and acknowledgements

Stomach content analysis provides many important information on ecological and biological aspects of fish behaviour, condition, habitat use, energy intake, inter- and intra-specific interactions. It is an essential part of the ichthyological research, fishery, and fish protection. Thus, the knowledge in the gut content analysis of fish is often necessary not only for ichthyologist, but also for other specialists in freshwater ecology, and employees in fishery and aquaculture. Moreover, researchers focusing on aquatic macroinvertebrates, zooplankton, algae, cyanobacteria and other experts focused primarily on different groups of aquatic, semi-aquatic or riparian organisms often help to ichthyologists, fish ecologists and fish farmers in diet analysis. They have the advantage of the knowledge of the prey (fish food) identification and the availability of potential food particles estimation. They are skilled in hydrobiological methods of sampling and quantifying of the density or biomass of potentially available food. Some of them become more experts on fish food than on the primary research from time to time. Sometimes, however, they are struggling with a lack of knowledge on the methods of gut content analysis, quantifying of food particles and methods evaluating initial results. Ichthyologist and fish ecologist starting work in the field of fish feeding using gut content analysis also need to study this specific research area. Numbers of reviews, books and papers on methodology were published, but there is a lack of a comprehensive source of information. They remain scattered and sometimes contradictory. Some sources of methodologies are too general and do not offer a detailed protocol or step-by-step instructions. Therefore, it is challenging and laborious to obtain a clear view on the fish feeding analysis prior to the research itself.

For the above reasons, this scientific monograph deals with the topic of the stomach content analysis of fish to help to present practical guidelines on individual procedures starting with sampling, through processing, identification, quantification, to evaluating and interpretation of data obtained. We outline qualitative and quantitative techniques used to describe food habits and feeding patterns of fishes and detailed description of particular methods of the direct stomach content analysis. For a better understanding of diet data, and for accurate interpretation of fish feeding patterns, we summarise and discuss pros and cons, possibilities, applications and limitations of different methods to provide a guide for potential users how to choose the method appropriate for the examination of particular details of fish feeding ecology.

We start with general information but concentrate more on methods appropriate for the freshwater fish study. Even though the methods are essentially the same in freshwater and marine species, but there are some differences related to the environment, study scale, sampling methods, sampling size, resources, diet composition etc.

This publication is intended for specialists in freshwater ecology, fish biology and ecology, employees in fishery and aquaculture.

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Introduction

Central to the study of animal ecology is the usage an animal makes of its environment (Johnson 1980). Animal populations need adequate quantities of usable resources to sustain and one of the most fundamental questions in ecology is what resources a particular species requires to exist (Litvaitis 2000). Therefore, it is necessary to identify the resources used by animals and document the availability of those resources. Besides the natural interest in animal ecology and thirst for knowledge, such documentation is critical in efforts to preserve endangered species and manage exploited populations (Manly et al. 2002; Simpfendorfer et al. 2011). The essential resources are food which animal consumes and the varieties of habitats which animal occupies (Johnson 1980). The knowledge of diet composition and feeding habits is, therefore, an important introduction to the natural history of any species (Ahlbeck et al. 2012; Litvaitis 2000).

Food habits of different species have been investigated for a variety of specific reasons important in a broader sense. Knowledge of natural diet in an animal species is generally essential for studies of animal nutritional requirements and the recruitment dynamics within a species and across various habitats to understand trophic, material and energy dynamics and to model outcomes for all ecosystems (Cutwa & Turingana 2000; Jordán et al. 2006; Navia et al. 2010). Data on feeding ecology can be used to construct food webs and predict possible changes in food chains and material and energy transfers between and within ecosystems (Nakano & Murakami 2001; Baxter et al. 2004, 2005; Rezende et al. 2008). It helps us to explain interactions with other organisms - potential competitive interactions among sympatric and predator–prey interactions species (Williams 1981; Jaksić et al. 1993). Information on the diet also contributes to the understanding of ecosystem structure, community composition and population dynamics (Ahlbeck et al. 2012; Litvaitis 2000).

In ichthyology, fish ecology and fisheries, the information on diet and food habits are valuable in the decision-making process related to natural resources (Kido 1996), quantifying the thread of an introduced or even invasive fish species to native fish populations (Fritts & Pearsons 2004). Moreover, this information is also important in assessing ecosystem integrity and assemblage functional redundancy (Matthews et al. 1982), understanding of such subjects as resource partitioning, habitat preferences, prey selection, and developing conservation strategies. It is, therefore, a key element in the protection of species and ecosystems, understanding the natural history of a species and its role in the trophic ecology of aquatic ecosystems (Braga et al. 2012).

Consequently, the study of the gut content is not only way to know the diet but also superior source of information on many aspects of fish biology and ecology. We will discover reasons to study stomach contents in more detail and particularly related methods in following chapters.

1 Why study gut contents?

What can stomach content help us explain?

Stomach content analysis[1] provides important insight into fish feeding patterns. Feeding of fish represents an integration of many important ecological components that includes behaviour, condition, habitat use, energy intake and inter- and intra-specific interactions, etc. Accurate description of fish diets and feeding habits also provides the basis for understanding trophic interactions in aquatic food webs. Conceptually, trophic relations of fishes begin with food and feeding behaviour of individuals or species. Diet composition analysis can be used to evaluate effects of ontogeny or the establishment of exotic species (Gelwick & Matthews 2006; Chipps & Garvey 2007). The history of gut content analysis is characterised by a shift from a basic description of food habits towards a more complex approach using information on feeding habits in new fields of scientific research. The simplest purpose is to determine the most frequently consumed prey or determine whether a particular food category is present in the stomach. But, one may be interested in more complex questions. The gut content analysis makes possible to answer questions, such as determining the relative importance of different food items to fish nutrition, quantifying the consumption rate of individual prey items, or understanding foraging trade-offs associated with predator avoidance. Assessment of food habits is also an important aspect of fisheries management and our ability to manage prey resources, increasing fish production and manipulating forage fish populations to enhance sports fisheries (De Vries & Stein 1990; Kamler & Pope 2001; Pikitch et al. 2004; Chipps & Garvey 2007). In summary, stomach content analysis is used in the understanding of many aspects of fish ecology on individual, population, community and ecosystem levels. It helps us study and explain specific problems of interactions, evolution, speciation, invasions and fishery management nature protection. As a result, stomach content studies can be incorporated into a variety of different research objectives. Specifically, the real application involves a range of topics, such as:

- prey selection (e.g., Kohler & Ney 1982; Stergiou & Fourtouni 1991; Adámek et al. 2004; Isaac et al. 2012; Ranåker et al. 2014);
- predator-prey size relationships (e.g., Scharf et al. 2000; Jennings & Warr 2003; Hartvig & Andersen 2013; Nakazawa et al. 2013);

[1] Methodological note: The alimentary tract can be divided into anterior mouth, buccal cavity, pharynx and posterior regions. The posterior part consists of the foregut (oesophagus and stomach), midgut intestine, and hindgut (rectum). It is important to note, that many fishes lack true stomachs. Also the length of the intestine largely varies in fish. It correlates generally with feeding habits (from 1/3 to 1/4 of the body length in some carnivores to 2 to 20 times the body length in herbivores and detritus feeders (Helfman et al. 2009). It is easier to divide the gut into segments and take decision on what sections will be analysed in fish with true stomach and short intestine straight or U shaped intestine. It could be more difficult to do it in herbivorous fish without a true stomach and with a long intestine.

- community structure (e.g., Jennings et al. 2002; Wilson & Wolkovich 2011);
- interspecies an interactions such as competition (e.g., Macpherson 1981; Robinson & Wilson 1994; Adámek et al. 2003, 2004; Svanbäck & Bolnick 2007; Araújo et al. 2008; Crow et al. 2010; Ševove & Kováč 2013; Leduc et al. 2015);
- territoriality (e.g., Bo et al. 2010);
- consequences of interspecies interactions (e.g., Kido 1996; Adámek et al. 2004 Olden et al. 2004);
- niche overlap and niche partitioning (e.g., Bellwood et al. 2006; Herder & Freyhof 2006;Longenecker 2007; Quevedo et al. 2009; Crow et al. 2010; Guzzo et al. 2013; SA-Oliveira et al. 2014; Córdova-Tapia et al. 2015);
- ecotypes coexistence (Hartvig & Andersen 2013);
- trophic level (e.g., Stergiou & Karpouzi 2002; Pauly & Watson 2005);
- food web structure (e.g., Garvey et al. 1998; Vander Zanden & Vadeboncoeur 2002; Layman et al. 2005; Quevedo et al. 2009);
- trophic cascades (e.g., Eby et al. 2006; Heithaus et al. 2008; Frid & Marliave 2010);
- connections between terrestrial and aquatic food webs (e.g., Nakano & Murakami 2001; Baxter et al. 2004, 2005; Rezende et al. 2008);
- parasite – host relationships (e.g., Barber et al. 2000; Knudsen et al. 2004);
- the association between fish food resources and its morphological traits (i.e., a phenotype-environment relationship, intraspecific resource polymorphism) (e.g., Robinson & Wilson 1994; Wimberger 1994; Hegrenes 2001; Svanbäck & Eklöv 2002; Kahilainen & Østbye 2006; Svanbäck et al. 2008);
- mechanisms for adaptive radiation (e.g., Schluter 1996; Cooper et al. 2010);
- the estimation of fractional trophic levels (TROPHs) which is essential for the management of fisheries resources as well as for quantifying the ecosystem effects of fishing (Stergiou & Karpouzi 2002);
- ontogenetic diet shifts (e.g., Stergiou & Fourtouni 1991; Labropoulou et al. 1997; Cocheret De La Morinière et al. 2003; Pilatti & Vanni 2007; Nunn et al. 2007; Costalago et al. 2012; Ševove & Kováč 2016);
- non-indigenous and invasive species impacts (e.g., Stergiou 1988; Grabowska & Grabowski 2005; Eby et al. 2006; Koščo et al. 2006; Musil & Adámek 2007; Koščo et al. 2008; Grabowska et al. 2009; Polačik et al. 2009; Reshetnikov et al. 2013; Ševove & Kováč 2016);
- hydrological conditions and water level fluctuation impacts (e.g., Bo et al. 2011);
- conservation strategies, protection of species and ecosystems (e.g., Pusey & Arthington 2003; Hoggarth et al. 2005).

Studies of fish feeding ecology are also used to test hypotheses and predictions based on:

- optimal foraging theory (Werner & Hall 1974; Sih & Christensen 2001; Wootton 2012; Svanbäck & Bolnick 2007);
- ecosystem modeling (Piana et al. 2006; Angelini & Gomes 2008);
- evolution of trophic adaptations *versus* phenotypic plasticity (Robinson & Wilson 1994; Skúlason & Smith 1995; Mittelbach et al. 1999; Collar et al. 2009);
- speciation (e.g., Wimberger 1994; Adams & Huntingford 2004; Snorrason & Skúlason 2004; Klemetsen et al. 2006).

2 Methods and approaches in feeding ecology of fish

How to investigate the food habits of fish?

There are different methods used for the fish feeding ecology research. All of them have both advantages and limitations. They can explain different questions or at least help us to understand different aspects of fish ecology and biology. We can classify several groups of methods. These groups use different approaches and data acquisition from neither invasive nor destructive contactless observations to invasive, sometimes destructive methods (sacrificing the examined individual). The following categorisation[2] is modified tablature of Litvaitis (2000):

Direct observations

Direct observation and video-recording have been widely employed in studies involving laboratory populations. These techniques are useful in studies of foraging behaviour (continuous recording allows assessments to be made of changes in feeding behaviour and foraging-site selection induced by the introduction of competitors or predators), in the investigation of the feeding responses to different food items, for the study of feeding behaviours in response to different forms of food delivery, in studies designed to examine the influences of temporal or spatial clumping of food on food acquisition and the abilities of different fish to defend and monopolize food supplies (see Jobling et al. 1995). Direct observation has been used to study feeding for several decades under experimental conditions and in the field. Most common is this method used in small experimental units with small numbers of fish, but also in aquaculture tanks, stream channels, sea cages, and natural waters. Compared with other methods, it is possible to monitor individual feed intake on a minute-to-minute scale. Detailed descriptions of feeding behaviour and social interactions and a combination of different measurements on individual fish enable elucidation of mechanisms not possible when using other methods (Jobling et al. 2001).

The technique of direct observation is hampered by several limitations; limited to species that occupy open habitat; forage during daylight periods. It is also restricted to reasonably clear water. For nocturnal observations and in those regions where light levels are low, artificial illumination may be necessary and this can introduce a bias that is difficult to evaluate. Moreover, the presence of the observer is usually a disturbing influence often leading to behavioural changes, an alteration of feeding behaviour and foraging pattern. Nevertheless, direct, in situ observations yield data that are far closer to the undisturbed state than either laboratory (aquarium observations) or indirect sampling methods (Smith & Tyler 1973; Jobling et al. 2001). Direct behavioural observation, including the

[2] The *'on-demand' feeding systems* are not involved in the categorisation. Methods based on these systems are used only for fish trained to operate 'on-demand' feeding devices and restricted to pellets feeding.

feeding behaviour of wild fish in nature, is extremely time-consuming, expensive and difficult to apply to large-bodied or cold-water species of fish that occur outside of the littoral zone because low temperatures also multiply the difficulty of spending sufficient time in the water to obtain meaningful information. Additionally, without a large sampling effort, observation-based methods cannot offer long-term average estimates of activity costs for populations of fish. Like all other types of data, field observations have to be repeated a sufficient number of times to establish statistical limits and to be certain that they do not represent any anomalous condition. They have to be interpreted in accordance with rigorous, logical procedures just like any other observational or experimental data (Smith & Tyler 1973; Jobling et al. 2001; Rennie et al. 2005).

Feeding behaviour and food consumption in captive populations of fish is easier to observe, but this kind of methods is usually confined to the study of fish held in small groups. This is the reason, why it may be difficult to generalise the results to the conditions experienced by fish in commercial aquaculture where single fish may behave differently (Jobling et al. 1995, 2001). In laboratory conditions, the observer takes notes of the feed intake and feeding behaviour from behind a masking screen, or from a darkened adjacent room (Jobling et al. 2001). The limitations of these methods are artificial conditions which often cause alternation of fish behaviour. It is much more applicable to the study of captive fish than in wild fish removed from natural biotope.

Recent developments of video equipment and within computer programming enable detailed studies of feed intake, feeding behaviour, social interactions and swimming patterns (Talbot 1985; Jobling et al. 1995, 2001). The use of remote video has been widely used for example in reef ecology. It offers some advantages for experiments that would otherwise require long periods of underwater observation and also eliminates some of the limitations and shortcomings of direct observation. This approach allows feeding to be quantified in the absence of divers (Longo & Floeter 2012; Mendes et al. 2015) and enables detailed studies of feed intake, and feeding behaviour to be undertaken. More detailed studies may include registrations of frequencies of orientations towards food items, approaches towards the food, frequencies of rejections, patch choice and social interactions, including frequencies of aggressive behaviour such as displays, nips, attacks and displacements (Jobling et al. 2001). There are also sophisticated methodologies of recording in the laboratory to measure and analyse different behavioural endpoints (e.g., Brännäs & Alanärä 1992; Kane et al. 2004).

Direct observation and video recording are sometimes combined with external tags (Jobling et al. 2001) or PIT tagging to obtain more complex information (e.g., Meynecke et al. 2008).

Exclosures

This group of methods is usually used for the long-term effect study. It is the comparison of used sites and sites where access has been restricted by an exclosure. Exclosures are usually cages that limit access by fish but do not affect the plants and invertebrates. Exclosures can reveal information on general food habits of herbivorous and benthophagous fish when based on short-term

differences between paired (fenced and open) plots. The most common use of exclosures is to reveal the effects of fish on plankton, aquatic plant and benthic community composition, diversity, density and biomass (e.g., Van Donk & Otte 1996; Dahl 1998; Marklund et al. 2002; Ruetz et al. 2002, Baylei & Li 2008; Iglesias et al. 2011).

Postingestion samples

This is the most common technique for analysing food habits. It is primarily a method for qualitative estimation of the dietary composition by investigation of prey items in the fish stomach, but researcher often assess also the quantity of food. These methods involve sampling either during or after the digestive process. Contents of alimentary tracts are generally collected sacrificing the animal. Emetics, flushing tubes, and other similar techniques have been also used, to purge the upper portion of the digestive tract without harming the fish. Accurate data on the measures, mass, sex, age, body condition (and fertility, ploidy, parasites, etc. when the fish are killed) of the sampled fish and information on the quantity of prey consumed is an advantage of this group of methods. They are most often used in field studies of fish ecology. Reviews and summaries of these methods are given, e.g., by Hynes (1950), Horoszewicz (1960), Hyslop (1980), Talbot (1985), Cortés (1997), Kamler & Pope (2001), Ahlbeck et al. (2012). These methods use primarily the direct (microscopic) analysis of the gastrointestinal content. (We will deal with this group of methods in detail in the following chapters.) Later, it was possible to identify partially digested prey using rarely used methods such as biochemical signatures and modern approaches of food identification such a stable isotope analysis or DNA analysis are also available and used in specific research projects focusing on feeding ecology and trophic interactions (e.g., Hartman & Garton 1992; Teletchea 2009; Valentini et al. 2009; Carreon-Martinez & Heath 2010).

Specific methods of X-Radiography (X-Ray imaging), radioisotopes, dyestuffs and chemical markers

X–Radiography was adapted for studies of fish feeding and digestion (the degradation of X-ray-dense skeletal elements of the prey, and their passage through the gut of the predator). The spiking of soft-bodied prey with X-ray-dense contrast medium permitted qualitative studies of digestion and evacuation. The replacement of the dispersed contrast medium with X-ray-dense particulate markers opened the way for the quantitative determination of gastrointestinal content. The method is based on providing the fish with feed containing particulate X-ray-dense markers. The amount of marker eaten is usually measured by X-raying the fish, and counting the numbers of marker particles present in the gastrointestinal tract. When the X-radiographic technique is used for the quantitative estimation of feed intake by individual fish, the particulate marker must be retained within the gastrointestinal tract for some time. Any defecation of marker that occurs in the period between the start of feeding and the time at which the fish are X-ray photographed will lead to feed intake being underestimated. These methods were almost exclusively

carried out in laboratory conditions only. This approach is also better suited for studies carried out using unnatural prepared feeds than those in which natural food is fed. Moreover, these methods have more limitations and are restricted practically to study of digestion, feed intake and feeding responses of individual fish (see Jobling et al. 1993 and more references in Jobling et al. 2001). Recently, the digital X-ray imaging was evaluated again as a non-invasive method for examination of stomach contents of small fishes (Beckmann et al. 2015). The results of this survey indicate that for certain study goals, X-ray radiography may provide a time reducing, non-invasive technique for diet analysis of small fish. Based on both a feeding experiment and examination of field-collected preserved specimens, Beckmann et al. (2015) found out that digital radiography consistently revealed the presence of moderate- to high-density prey items in the stomach, such as small arthropods. Moreover, X-ray imaging allowed for the rapid identification of some particular prey items such as detritus, dipteran larvae, ostracods, hard-shelled molluscs, and small fish. Digital X-ray images can be also quickly acquired from anesthetised or preserved animals (30 seconds per fish), permit rapid identification of certain prey items and facilitate digital data archives. Also Adámek et al. (1990) successfully used this method in the determination of food passage. These new results and modern technological possibilities indicate great promise for the future for this group of methods and the elimination of some limitations.

Radioisotopes

This rarely used method is based on the incorporation of radioisotopes into the diet which is then fed to the fish. The radioactivity from the ingested feed present in the gastrointestinal tract is measured and feed intake estimated by reference to the amount of radioactivity added to the feed. The radioisotope method may be suitable under some conditions, but it does suffer from several disadvantages, such like the health point of view, and considerable care is needed to limit the risk of loss of isotope to the environment (see Jobling et al. 2001 for more details).

Various *dyestuffs and other chemical or biological markers* have been added to fish food for the study of digestion and rates of gastrointestinal transit. These methods have not gained popularity for measurement of feed intake in fish (Jobling et al. 2001; Adámek et al. 2011) nor in other aspects of fish feeding ecology.

3 Direct stomach content analysis in feeding ecology of fish

The study of the feeding habits of fish and other animals based on analysis of stomach content has become a standard practice many years ago (Hyslop 1980). Recently, several other methodologies are currently being used, such like above mentioned radioisotopes, stable isotope analysis, direct observations, and fatty acid analysis (see more references in Braga et al. 2012). These methodologies have positives (they are more precise and can reveal also items which cannot be identified by microscopic analysis) and negatives (expensiveness, complicated procedures). Nevertheless, the direct gut content analysis carried commonly out through dissection or evacuation and examination of gut contents is still the most used and easiest method with great potential and good enough for most ecological studies. In this chapter, we will describe the process of the study design preparation, some aspects of sampling, gut content acquisition, material processing and prey identification (Fig. 1). In the end, we briefly list the possibilities of some modern methods in food items identification.

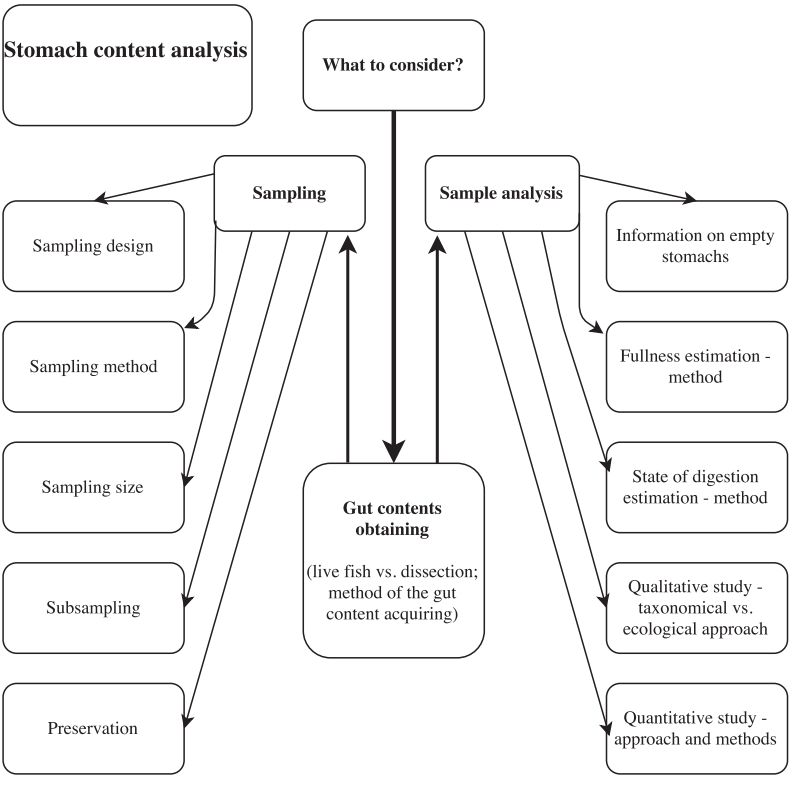

Figure 1 Scheme of important steps in the process of direct stomach content analysis planning. (See next chapters for explanation and description of individual steps.)

3.1 Designing an appropriate sampling designs for field studies

For better understanding of diet data, and for accurate interpretation of fish feeding patterns, many important aspects of the sampling design need to be considered before initiating a diet study. Moreover, different questions about fish diets necessitate different approaches in how one collects and analyses the data. The scale of the study - size of the area to be studied - needs to be determined. This area must be of sufficient size to adequately reflect the true nature of the target population or community. However, if it is too large you will waste resources and may not be able to complete the study. Whether the area of study should be a single habitat or selected representatives of the habitat type from a wider geographic area will depend on whether an intensive or an extensive study is planned. Extensive studies have a low intensity of sampling per unit area or through time, intensive studies involve the repeated observation of the population of an organism with the intention of producing accurate estimates of target parameters. The sampling design should be also well evaluated before data are collected. Appropriate sampling designs for diet analysis include (1) simple random sampling, (2) stratified random sampling, (3) systematic sampling, and (4) multistage sampling. The choice of a particular sampling design depends on a variety of factors that include the research question, logistics, accessibility, and costs (Chipps & Garvey 2007; Henderson 2009). Attention should be given the time of sampling. Foraging behaviour of fishes often varies with time of day (Shepherd & Mills 1996). Hence, sampling plans should incorporate a diel feeding chronology (diurnal feeding activity). The timing of sampling is important also from the other point of view. If the measurement times among sites differ, it may lead to erroneous conclusions about foraging patterns. Also the length and starting time of the interval yield different results on diel feeding chronology (Cortés 1997; Chipps & Garvey 2007). Diurnal changes in diet composition in combination with unsuitable timing may considerably bias food consumption estimates for fish species that feed both on animal prey and plant material (Horppila 1999). Sampling gear can affect results for diel feeding chronology and food consumption estimates. Active gears, such as electrofishing, beach seines, and trawls are used for representative samples, preferably at regular intervals over 24-h periods. For large fish that can evade active gear, passive gear (e.g., gill nets) is usually set over a similar time period, but retrieved more frequently (Gelwick & Matthews 2006). Different gear used for the same fish population can lead to wrong feeding activity assessment. It affects inferences about stomach fullness because passive gear only effectively samples those individuals in a population that are actively feeding. Consequently, the amounts of food in stomachs are higher than in samples collected using active gear because it samples both low-activity or non-foraging fish and actively feeding fish (Hayward et al. 1989). Moreover, it should be taken into account that different sampling approaches may cause different values of the stomach contents loss through regurgitation (Bowen 1996). Activity and feeding are likely to be influenced by a large number of environmental variables. Environmental control of feeding behaviour caused by temperature, light level, turbidity, current, barometric

pressure, wind and turbulences (see review by Stoner 2004) can consequently significantly affect the results of research. This implies that these environmental variables must be recorded (Henderson 2009) and reflected in the analysis and data interpretation.

In most cases, the researcher also needs a sample of potentially available food. We discuss the topic of availability in another chapter. A sampling method of organisms and other biological matter in the environment is a very broad topic and it is even not possible to outline the basics in this publication. The sampling technique depends on the environment, target food source (prey), its availability and many other factors. After clarification of what actually is an available food source for studying fish in a particular environment, we propose to study dedicated information sources, methods and techniques designed to gain appropriate samples of these organisms or organic matter.

For more detail information on the sampling design, it is very appropriate and desirable to study the general ecological and limnoecological principles, guidelines and reviews of sampling design planning (e.g., Cortés 1997; Brower et al. 1998, Henderson 2009; Chips & Garvey 2007; Hauer & Lamberti 2011).

3.2 Sampling size

Many tutorials and reference books highlight the rule: “Never undertake an ecological study without considering how many samples will be required to meet your objective”. Obviously, it is better to know how many samples are needed to describe the diet before initiating the field study. It is possible to answer the question “how many samples are needed to describe the diet” using cumulative prey curves. They are useful for determining the sufficient number of sampled stomachs. In this approach, the cumulative number of prey items is plotted against the cumulative number of pooled stomachs. The point at which the curve becomes asymptotic provides a minimum number of stomachs needed to characterise prey composition (Cortes 1997). Other techniques are also available, such as the power analysis for which was the free software G*Power developed (Faul et al. 2007, 2009). The question about the validity of inferences raises when sample sizes are random variables. This case is not rare in practice. Researchers often take whatever sample size they can get (Manly et al. 2002). The sample size can be limited by the population size, the density of individuals, the conservation and/or endangerment status of species, financial capabilities and resources. Anyway, it is desirable to consider the sample size and obtain at least comparable datasets and thus more reliable results if it is not possible to reach the optimal sample size.

3.3 Subsampling across size-classes

When large numbers of fish are encountered in the field study, the subsampling across size-classes is required. The stratification of samples as a function of body size is important because the size of individual often affects both the quantity and quality (composition) of items within the diets of particular

species. The diet may differ slightly in some species, but others may belong to different feeding groups at different stages of its life (e.g., Dunbrack & Dill 1983; Byström et al. 1998; Lundvall et al. 1999; García-Berthou 2001; Rezsu & Specziár 2006; Koščo et al. 2008; Nunn et al. 2012). The number of subsamples taken can reflect (1) the relative proportion of individuals within each size-class, (2) some set number of individuals per size-class. Subsamples taken randomly in proportion to the actual number within each size-class reasonably reflect size-based patterns within the whole sample. These sampling designs may poorly represent the diets of the infrequent largest individuals within the population. To avoid this, sampling designs which incorporate the set number per size-class approach are also used (Chipps & Garvey 2007).

3.4 Live fish, ice, ethanol, or formaldehyde preservation?

Stomach contents can be collected either from the live, fresh died or preserved fish or alimentary tracts. The actual mechanics of analysing samples is rarely considered, but they are likely to affect diet results. Most diet samples are not analysed forthwith. They should be preserved immediately either by freezing or by fixing to avoid continued digestion (Chipps & Garvey 2007). Each of these preservation techniques essentially combines the advantages and disadvantages. Apart from the different demands on equipment, financial costs, health or environmental risks which we do not deal with, there are also differences in the effects on preserved materials. This is a really serious problem which can make impossible to compare results from different studies and even correct evaluation of preserved samples with prey of different size using gravimetric methods. Assessing prey mass using the length-mass relationships can be also affected by changes due to preservation. It is also important to take into account those consequences if results are to be compared with previously published papers. For example, mass losses of food are well described by invertebrates (prey of predacious fish). Their mass loss and size changes due to the use of preservatives are often reported in the literature (e.g., Mills et al. 1982; Leuven et al. 1985; Heise et al. 1988; Shields & Carlson 1996; Kapiris et al. 1997; von Schiller & Solimini 2005; Paradis et al. 2007). The ethanol preservation generally results in mass loss through leaching (Leuven et al. 1985; Heise et al. 1988; Johnston & Cunjak 1999; von Schiller & Solimini 2005). The length-mass equations for animals preserved in ethanol predicted lower masses than those calculated for non-preserved specimens in the study of González et al. (2002). Such differences increased with animal size and cause underestimation of biomass. The effects of freezing on invertebrate mass and morphology are unknown in invertebrates. However, freezing in water causes both length reduction and dry mass loss in larval fish (Johnston & Mathias 1993). Thus, the effects of freezing on length and mass of invertebrates may also be problematic. The formalin preservation results in insignificant (e.g., Leuven et al. 1985; von Schiller & Solimini 2005) or minimal (e.g., Heise et al. 1988; Paradis et al. 2007) mass loss through leaching of organic matter, even an increase in mass (weight) caused by formalin preservation was also recorded (Parker 1963; Hyslop 1980). In contrast to most results, Wetzel et

al. (2005) did not record significant differences between the alcohol and formalin preservative treatments when comparing the wet mass, dry mass, and ash-free dry mass. In any case, according to the vast majority of papers, the effect of formalin on the biomass appears to cause smaller mass change than alcohol (Mills et al. 1982; Leuven et al. 1985; Puigcerver 1997; Paradis et al. 2007; Knapp 2012). On the other hand, formalin storage may lead to the dissolution of bones and otoliths of vertebrates (Jobling et al. 2001). They are used to estimate the age and size of partly or almost completely digested prey found in the stomach and the formalin based preservation can make that impossible. Many factors such as the span of storage or temperature can also affect the rate of changes in different preservation fluids and taxonomic variation in the mass loss and length changes is also not omissible (e.g., Paradis et al. 2007). It seems, that the combination of formaldehyde as the preservative fluid and freezing as the storage method is an optimal combination for studies in which the body mass of insects as the dominant diet component is considered and samples are stored longer time in the preservation solution (Knapp 2012). This is probably also the case for other groups of prey. The researcher should know how can fixation affect results before choosing this preservation medium and choose the preservation method following the scope of the study (Tab. 1).

Table 1 Suitability of different methods of fish and prey preservation for different analyses.

suitable for: / preservation	length-mass	mass	bones+otoliths
live fish	YES	YES	YES
ice	NO	NO	YES
ethanol	NO	NO	YES
formalin	YES	YES(?)	NO
formalin+freeze	YES	YES	YES

When it is impossible to handle with live fish, sampled fishes are usually preserved in 10% formalin or 70 % alcohol after sacrifice with an overdose of anaesthetic, for example, Clove Oil, a natural anesthetic or other anesthetic solution (e.g., Anderson et al. 1997; Keene et al. 1998; Sladky et al. 2001). When preserving fish heavier than 100 g, formalin or alcohol should be injected into the gut area. Alternatively, the abdominal cavity can be partially opened by carefully cutting with scissors to ensure the transition of preservation solution into the digestive tract and prevent further digestion of prey. When fish are not chemically preserved, it should be transported to the laboratory on ice, and then stored frozen until they are examined. The gut contents of piscivorous fish transported on ice can be preserved in saturated sodium bicarbonate solution. Very large fish should be dissected in the field. The digestive tract is then labelled and transported separately to accelerate its freezing.

3.5 Fieldwork and sampling – general basic rules

In the field, the researcher should follow the appropriate manuals, procedures, instructions, and safety rules of sampling. They are presented in countless publications (e.g., Holčík & Hensel 1972; Bain et al. 1985; Nickum 1988; Bohlin et al. 1989; Meador et al. 1993; Friend et al. 1994; Appelberg et al. 1995; Appelberg 2000; Nickum et al. 2004; Furse et al. 2006; Gelwick & Matthews 2006; Kubečka & Prchalová 2006; Jenkins et al. 2014). It is necessary to complete the field protocol responsibly. Writing down the site name (number), date, time and all important information, measures, characteristics and remarks in the prepared field form precede sampling. It is also appropriate to take photographs of the sampling site and record the GPS coordinates. The sample must be labelled immediately, unalterably and durably. Researches have to comply ethics rules when handling and killing fish. Prompt preservation or freezing fish keeps the digestive contents in conditions equal to the moment of capture and is necessary to prevent loss of resolution.

3.6 Analysis of fish gut contents using dissection

Dissection method is still the most effective and most precise results bearing method despite the fact that it is controversial in term of ethical, protection and economic issues. This method also makes possible to retrieve significantly more information on analysed fish. In addition, the relative poor recovery rates of food using some non-lethal methods, limitation by fish size, costs and time consumption in others are the reasons, why researchers still often select dissection when analysing the stomach content.

3.6.1 Sample preparation

All samples being analysed must be properly prepared (Fig. 2). Formalin is hazardous and must be neutralised before analysts can examine the stomach. Samples should be washed, gloves, safety glasses, and a lab coat are worn when samples are analysed.

Laboratory form is prepared and all the necessary information on the sample are recorded. Morphological measurements are implemented and the body mass of analysed specimen is measured after drying the fish for 1 min between two pieces of tissue paper. Depending on the goals of the study and size of fish, we use different measurement methods and accuracy. The level of precision required, and trade-offs between accuracy, precision, and the time taken for the measurements often need to be balanced (Lourie 2003). Recording of total length and standard length to the nearest 1mm and mass (“weight”) to the nearest 0.1 g is usually good enough in ecological studies. Note that manipulations of specimens for some morphological measurements are difficult on rigidly preserved specimens, compared to fresh tissues and, thus, the precision and measurement may be affected. The last step of the sample preparation is usually the external visual examination and recording of special features such like injuries, amputations, ectoparasites, etc. These can be valuable when inspecting

data before analyses and can help explain extreme values in some cases. We can also use them as variables in statistical analyses and evaluate their impact on the diet.

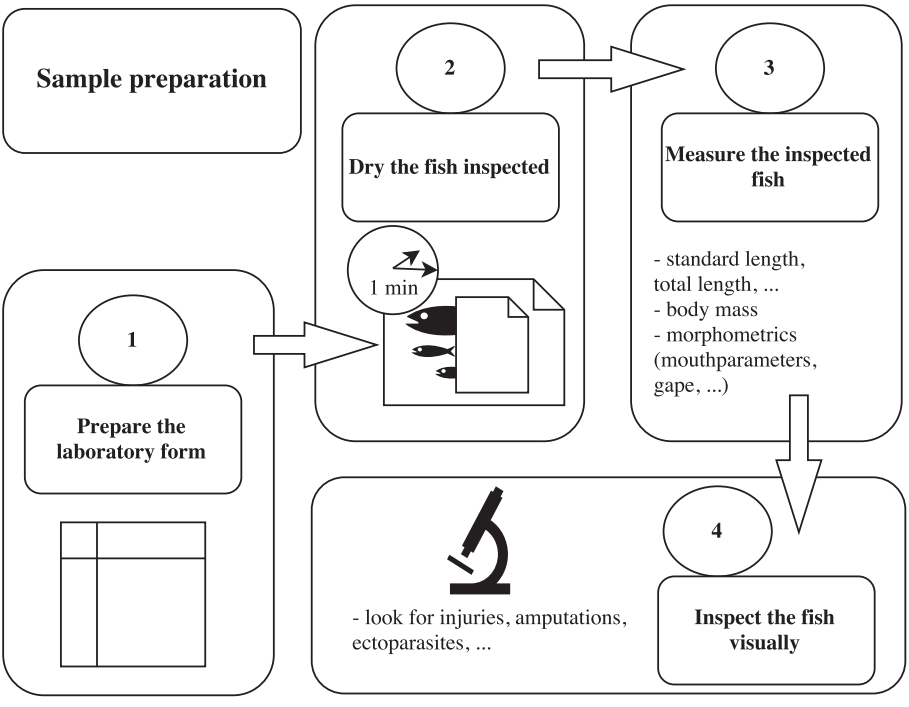

Figure 2 Schematic flowchart of the sample preparation.

3.6.2 Obtaining gut contents

This step is critical for obtaining appropriate material. Whereas this publication focuses on an analysis of gut content, we will describe the individual steps in the form of rigorous laboratory guide in this chapter (and also when describing methods of the gut content analysis of live fish). Following this step by step protocol, researchers will obtain an optimal gut content sample for fish-feeding research from the ichthyological material. Some steps e.g., length and/or mass of the gut may be omitted if they are not necessary for further analyses. Anyway, be aware that if you will perform these measurements, you can use them or not but if you will skip these steps, it will be impossible to obtain these data later. Assessing the need for separation of the stomach (foregut) as described in step 13 is highly recommended. Some authors suppose that investigators must consider the relative digestibility of prey when deciding on the section of the alimentary tract to analyse. Absolute or relative quantities of food ingested are difficult to measure and the food item is difficult to identify

when different food items are in a different state of digestion. This usually means that the degree of precision to which a natural diet can be analysed is low. To avoid bias when both easily digested prey and resistant prey are present, only the immediate foregut (i.e., stomach) should be analysed (e.g., Sutela & Huusko 2000; Liao et al. 2001; Baker et al. 2014 and many references in these papers). The best practice would be to analyse some guts first and evaluate the hindgut (intestine) content for further analysis. In any case, information on the content of different parts can help us better understand or add the supplementary data on the feeding activity. Immediate recording of all measurements, notes and remarks into the data table or laboratory form is necessary. Taking pictures of remarkable details or objects is recommended to put fish or the area of interest with a label on millimetre (graph) paper. As the food digestibility and diet are related directly to intestine length (e.g., Ribble & Smith 1983; Kramer & Bryant 1995; Herder & Freyhof 2006; Wagner et al. 2009), the measurement of the digestive tract could bring another important information for further study of diet in a biological context.

Material: scissors; scalpel; needles; tweezers; Petri dishes; stereomicroscope magnifier with micrometre; paper tissue or filter paper; electronic scale (balance); water; pipette; squirt bottle; laboratory form or data-table; pen; pencil; permanent marker; labels; tubes; plastic bags; preservation media; camera; millimetre paper

Method description (Fig. 3 and 4):

1. Using appropriately sized scissors or a scalpel, make a longitudinal cut on the ventral side of the fish from just behind the isthmus of the gills posterior to the anal fin.
2. Make two transverse cuts at each end of the first cut to open the coelom and expose the viscera.
3. Using sharp scissors, intersect the oesophagus, the last few millimetres of the intestine, and the mesentery at its dorsal point of attachment. This allows the visceral mass to be lifted out of the coelom for more detailed examination and manipulation.
4. Examine the abdominal cavity. (The special parasitological survey is the best solution.) Record each remarkable observation, collect, preserve and label parasites in appropriate preservation liquid (Huber 1998). Quantify and identify the parasites later.
5. Separate the digestive tract (oesophagus, stomach, and intestine) from other visceral organs.
6. Measure the mass of gutted fish.
7. Determine the development stage (juvenile vs. adult; age) and gender.
8. Examine the intestinal tract and other visceral organs. (The special parasitological survey is the best solution.) Record each remarkable observation, collect, preserve and label parasites in appropriate preservation liquid. Quantify and identify the parasites later.
9. Preserve and label other visceral organs for later examination (e.g., gonads for fecundity).
10. Clean the digestive tract and dry it with tissue paper.

11. Measure the length of the uncoiled dissected intestinal tract and length of its parts (foregut and hindgut or stomach and intestine) to the nearest mm. Use calliper or stereomicroscope with a micrometre. (Alternatively, you can use a digital camera and take a picture of the intestinal tract with label on millimetre paper to measure the length using appropriate software technique later.)
12. Measure the mass of the intestinal tract. Use electronic balance and record the results with precision to the nearest 0.001 g. (Alternatively, measure the mass volumetrically as described in another chapter.)
13. Sever the stomach (or foregut) from the hindgut. In fish, which do not have a discrete stomach, the anterior third or first half of the intestine can be dissected. (The stomach, foregut or the anterior section will contain the most recently ingested prey.)
14. Open the stomach or gut segment by making a shallow slit (be careful to not cut prey) lengthwise with fine scissors or a scalpel. Make a longitudinal incision avoiding damage to the contents, to reveal the food bolus. Use stereomicroscope for dissection of small fish's stomach.
15. Determine the gut fullness of individual gut sections using the method selected prior to the analysis. Record the corresponding code for the degree of fullness. (See more information on the fullness assessing possibilities in the dedicated chapters.)
16. Lift large prey items directly from the gut segment. For smaller prey, hold the slit segment with forceps over a Petri dish and wash out the contents with a small amount of water from a squirt bottle or pipette. The food also can be extruded by sliding a blunt probe along the length of the segment. But beware that this technique may extrude much of the gut mucosa as well. That should not be mistaken as part of the diet (Bowen 1996).
17. Dry (using tissue paper for one minute) and weight the empty gut sections to the nearest 0.001 g to obtain the mass of gut contents. The difference between the mass of the intact stomach and the mass of the empty stomach is the total mass of stomach contents.
18. Remove material that is obviously composed of parasites, stomach lining, mineral particles, or any other non-prey matter. Count these items or assess their portion in gut content. Measure the mass of this material to the nearest 0.001 g. (The mass will be not included in the food mass.) Note this information in the comments section of the laboratory form or data-table.
19. When analysing formalin preserved samples, keep gut contents in water on Petri dishes for at least five minutes (better for several hours or even a day) to remove excess formalin.

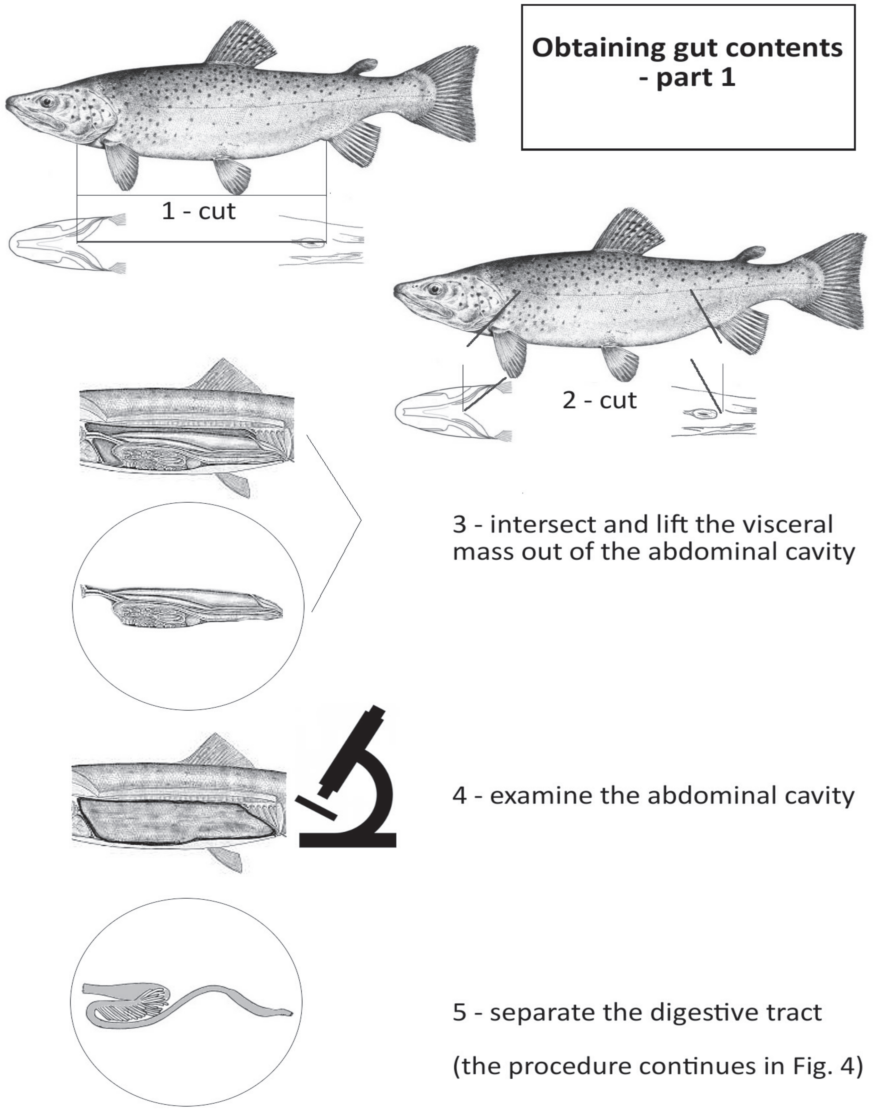

Figure 3 Schematic flowchart of the digestive tract preparation (continues in the Fig. 4).

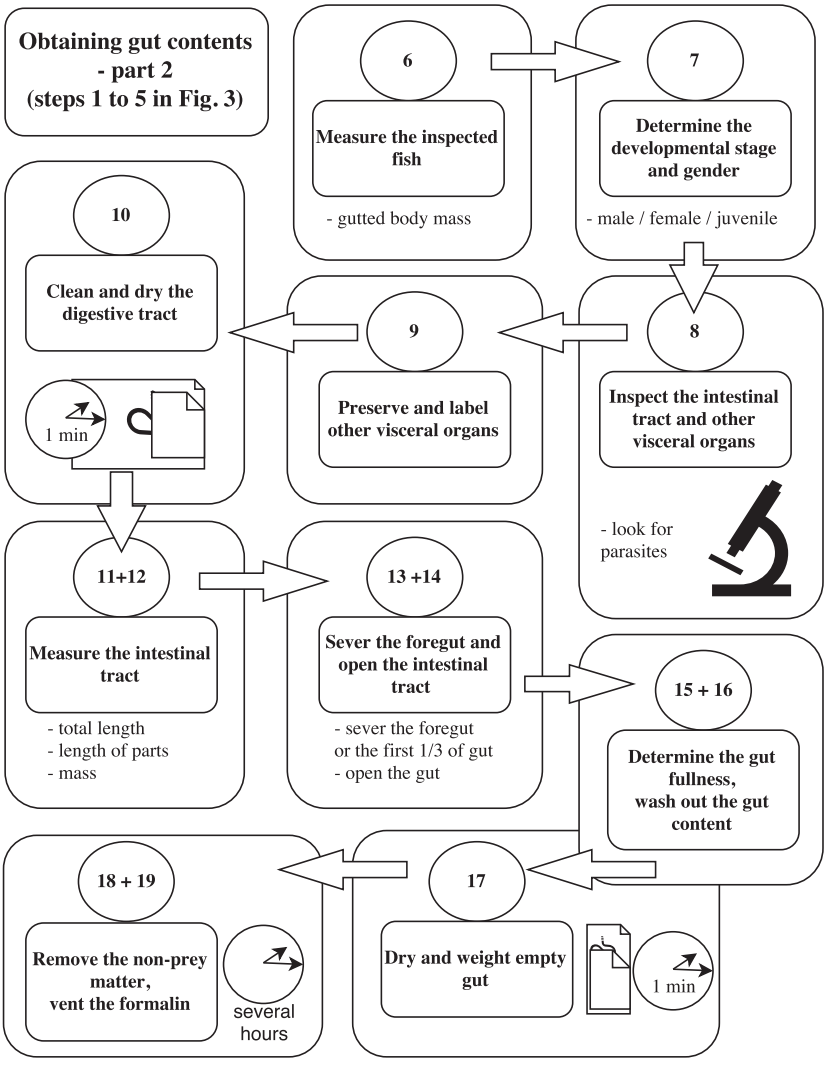

Figure 4 Schematic flowchart of the gut contents obtaining – gutted fish and digestive tract treating (continuation of Fig. 3).

3.7 Analysis of live fish gut content

Most studies have traditionally sacrificed relatively large numbers of fish to examine their stomach contents. However, sacrificing fish for food habit studies may cause public relations issues. They may not be a convenient option if study fish which are threatened, endangered, economically valuable, or come from a low-density population. Additionally, lethal methods may significantly alter the

population structure of fish. Accordingly, a number of nonlethal methods were developed to obtain and analyse the gut content of fish. They are based on the stomach contents extraction by mechanical means, or emetic substances (Hyslop 1980; Bowen 1996; Kamler & Pope 2001 and many references there; Elosegi & Sabater 2009). Nonlethal methods include the use of gastroscopes, tubes, stomach suction, stomach flushing, emetics, forceps, and chronic fistulas. Kamler & Pope (2001) review, describe, and compare these methods and report the effectiveness (ability to remove all stomach contents) of these methods. Some other authors published papers focused on the application and effectiveness of these methods later. We describe these techniques hereinafter with emphasis on the most efficient and most useful of them.

3.7.1 Techniques and devices

3.7.1.1 Gastroscopes

Gastroscopes are probably the simplest and least invasive of all the methods. The gastroscope is a long metal cone that is inserted into the mouth of a fish through the pharynx into the anterior part of the stomach. Also, acrylic tubes have been used as gastroscopes. The major food items in the stomach of the fish can then be visually discerned and recorded. Gastroscopes were also used to obtain food material from the stomachs, some authors used forceps in concurrence with gastroscopes. This method is inadequate for detailed analysis of the entire stomach contents and may be biased toward larger food items. It is also limited by feasible applicability in fish of bigger size. The effectiveness of gastroscopes to determine the diets of fish is uncertain (Kamler & Pope 2001).

3.7.1.2 Forceps

In this method, forceps is used to directly obtain the content from the fish stomach. Anaesthesia and concurrence with gastroscope have been used in this technique (Kamler & Pope 2001). Probably it can reduce the risk of tissue injury and increase the precision. Anyway, it would be inadequate to say that this method could be appropriate for any kind of ecologically focused gut content analysis. This method was used in only a few obsolete studies.

3.7.1.3 Emetics

Emetics are drugs or chemical solutions that induce regurgitation. A solution of arsenous acid, hydrochloric acid, tartar, hydrogen peroxide, apomorphine, or antimony are injected into the stomach. The fish are then placed in an aquarium and the stomach contents are collected from the bottom. The effectiveness of emetics is low if compared with other methods and even unsuccessful in some fish species. The use of emetics may have several other disadvantages. a collection of the stomach content in an aquarium is obviously a doubtful method of scientific data collection. The use of arsenous acid may leave trace amounts of arsenic in the fish and many of these substances are dangerous towards fish, environment and human health. Therefore, this method is only very rarely used in practice (Kamler & Pope 2001; Barbour et al. 2012).

3.7.1.4 Chronic fistulas

This curious method is not applicable in field research. The chronic fistulas were used very rare in the fish diet study. This technique is almost used for laboratory physiological experiments (e.g., Gay 2013).

3.7.1.5 Tubes

Glas tubes and acrylic tubes of suitable size are used to obtain stomach contents of fish. They are inserted into the gullet and the stomach content is forced out through the tube by exerted pressure over the stomach. Sometimes, it is necessary to inject water into the stomach through the tube first. The use of acrylic tubes appears to be relatively efficient in obtaining the stomach contents of fish. However, there are some limitations involving fish size, food size, and incomplete recovery. The tubes are most effective for larger individuals and least effective on fish with relatively small mouths and large stomachs. Highly variable is the recovery percent among fish species (50 – 100% in Kamler & Pope 2001; 0-100% in Quist et al. 2002) but almost all prey taxa are usually sampled disregarding some extremes. Thus, this method may be adequate for assessing the presence or absence of prey items in fish diet. The great advantage of this method is the minimal amount of equipment and the time required to obtain samples. Anyway, it is suggested that researchers should not rely solely on the data from tubed stomachs until the efficiency is not evaluated (Kamler & Pope 2001 and many references there; Quist et al. 2002). We recommend undertaking a pilot study for particular species or population as a comparison of the sample obtained by a tube with real gut content obtained by dissection of several individuals of different size classes. Then, this method can be advisable for some fish feeding ecology studies.

3.7.1.6 Stomach suction

A suction bulb, attached to a glass tube, is used to collect the stomach contents of fish in this method. The open end of the tube is inserted into the stomach of the fish through the mouth when the bulb is pressed. The pressure on the bulb is then released and the stomach contents are suctioned into the tube. This method is relatively simple. It is efficient in some species, however, it may be ineffective if the stomach contents of the fish are relatively large or if the study fish is relatively small. Additionally, the disadvantage of this method is that the technique of suction is laborious, and stomach contents may be damaged when transferring the suctioned contents into another container. A more complicated modification of this method was developed with an aspirator emptying the air out of a glass tube that is connected to a rubber nozzle. The rubber nozzle is inserted into the mouth of the fish and the continuous suction of uniform pressure facilitates the transfer of the stomach contents into the storage tube (Kamler & Pope 2001). This method is not well established in practice. Results of only few studies were obtained using suction. This technique does not offer benefits when comparing with the most used non-lethal techniques. Its efficiency is low in small species, in species feeding on large prey and it is unusable in large fish. The possible risk of tissue violation by vacuum is relatively high, so we do not recommend this method at all.

3.7.1.7 Stomach flushing

Stomach flushing, or gastric lavage, are a widely used technique for obtaining the stomach contents of live fish (Hyslop 1980). As likely in the above described methods, it is important to ensure that the removal technique effectively samples all items by flushing. Another way, the results may be skewed toward items that are more easily displaced from the stomach (Chipps & Garvey 2007). There are several different techniques and various types of equipment that are used to flush out the stomach contents of fish. Stomach flushing equipment has been categorised under hand pumps, mechanised pressure, or syringes, although there is some overlap with these groupings by Kamler & Pope (2001).

Handpumps

Using this device, two metal tubes of different diameters are soldered together and bent at the end for easy access into the esophagus of a fish. The opposite end of the larger tube is fitted into a bottle. Water or Ringer's solution is pumped through the smaller tube into the stomach using a rubber suction bulb with a check valve at each end for a unidirectional flow. The stomach content is washed through the larger tube into the attached bottle. Numerous variations of the original technique were developed such as india-rubber bulb with a glass tube (Andreasson 1971), semi-rigid polyethylene tubing held by epoxy glue (Gengerke et al. 1973), or fired-glass tubes allowing visual observation for completeness of pumping (Swenson & Smith 1973). Much smaller and simpler device for juvenile and other small fish were developed. A small Pasteur pipette is attached to a rubber tube leading to a small hand bulb equipped with one-way valves, and finally leading to a water reservoir. For fish with a simple s-shaped intestine and lack a pyloric sphincter, flushing the food items out the vent of the fish is used (see more in Kamler & Pope 2001). This method has been used successfully on a variety of fish species. Almost all researchers found this method very efficient with no influence due to the type of prey contained in the stomach (many references in Kamler & Pope 2001; Gelwick & Matthews 2006).

Mechanised pressure

Sprague et al. (1993) showed that water at too high pressure could lead to internal injuries or even death by rupturing the swim bladder. Because of that, techniques using controlled mechanised pressure were developed. An example of these innovations is pulsed gastric lavage, consisted of a hypodermic needle and a polyethylene tube, coupled with a water pump by a variable pressure valve and has the advantage of providing a continuous supply of water. The size of the needle and tube is adjustable to the size of the fish. The adjustable valve is then opened and closed to allow pulses of water under pressure to pass into the stomach. The water pressure flushes the stomach contents through the oesophagus and into the collecting container. Similar but slightly modified techniques include the use of a 12-volt portable pump or the use of hose clamps with a quick-disconnect fitting for greater speed when changing the size of the tubing. This method was evaluated for numbers of species of fish and found it to be very (sometimes 100%) effective with no mortality in some species, but

sometimes less effective in dislodging large food items or ineffective for removing the stomach contents of small specimens. The method caused high mortality and was ineffective at removing the stomach contents in one published case (Hartleb & Moring 1995). The advantages of this method are relatively low costs, ease of operation for one person, the efficiency of removing stomach contents, and durability and portability of the apparatus. Even if the control over the pressure is better, this technique can cause mortality. Hence, it is recommended only to sample larger fish (Kamler & Pope 2001; Brosse et al. 2002; Barbour et al. 2012).

Syringes

Syringes are often used to obtain stomach contents of relatively small fish when the apparatus from other methods is too large. This technique could obtain stomach contents by anal backwashing for fish species with no pyloric sphincter (Faina 1983), or by stomach flushing through the oesophagus for species with a pyloric sphincter. A short tube can be inserted through the anus into the intestine of the fish. Water is injected through the tube with a hypodermic needle and the gut and stomach contents are flushed out through the mouth. Another way how to use syringes is to inject water directly into the stomach of fish through the mouth and oesophagus. The stomach contents are flushed back through the oesophagus and mouth, through a funnel into a container. The more complicated device uses two sizes of syringes, a plastic tube, and interchangeable rubber tubes. Water is forced into the stomach through the oesophagus by the smaller syringe through a plastic tube which is encased within a rubber tube that lead to a larger syringe for the collection of the stomach contents. The diameter of the tubes and the volume (capacity) of syringes depend on the size of the fish. These methods have been evaluated as very effective removing up to 100 % of the stomach contents. Although syringes may be effective for some species of fish, they may be ineffective and even fatal in other species. Water pressures associated with the stomach flushing may probably harm swim bladders and cause other internal injuries in small or juvenile fish. Softer material (intramedic tubing) and careful handling, may perhaps reduce the mortality (Kamler & Pope 2001 and many references there).

3.7.2 The process of non-lethal sample collection

Anaesthetics, the Clove Oil solution (0.03 – 0.05%, with respect to fish species) for example, or another anaesthetic solution is used before starting the fish processing[3]. Complete loss of the fish's equilibrium signals appropriate anaesthesia. Wet hands should be used when fish is handled to avoid removing the protective mucous coating from the fish surface. Identification and recording of sex, life stage and reproductive state (if possible) is an important initiatory step. After that, morphological measurements are implemented and the body mass of analysed specimen is measured. Recording of total length and standard length to

[3] The captured fish is sometimes placed into a plastic jar (pre-labelled or put a label inside). The one-third full of ice-water containers are advisable to immobilize the fish. However, this technique is in contradiction with fish welfare principles!

the nearest 1mm and weight to the nearest 0.1 g is sufficient. An external visual examination and recording of special features such like injuries, amputations and ectoparasites follow. Digital photograph of the fish with a scaling factor and label is a proven procedure which maintains important information for possible future reference. Gut content inspection (gastroscope) or gut content obtaining is the crucial step which we describe below. Measure the mass of fish again to assess the mass of food. In the end, fish are transferred to plastic containers with continuously aerated water from the sampling site, until their full recovery and then returned in natural habitat. Recording the rate of mortality may provide useful information for further studies and help other researchers to choose the best method in future.

3.7.3 Obtaining gut contents

We describe the most often used techniques of passive and active stomach flushing below. These methods require a special apparatus and longer handling time and preparation, so fewer animals can be sampled if field time is limited. Gastric lavage can be affected by the stomach shape, the size of the mouth, and food items of particular fish species. Thus, reliability must be established for each species (Kamler & Pope 2001; Waters et al. 2004; Gelwick & Mathews 2006).

Passive gut flushing with tubes

Material: clear, smooth, nonflexible tubes (plastic or glass) of various diameters with bevelled ends to ease their entry onto the stomach; a trough with measure (mm scale); containers with water; funnel; digital camera; labels; permanent marker; pencil; containers

Method description (Fig. 5 and 6):

1. Wet the end of the tube before inserting it into the fish. For each fish, select the largest diameter tube that easily passes the oesophagus without injury to the fish.
2. Using wet hands, one person holds the fish oriented with its head and dorsal side upwards in the trough.
3. Another person inserts the wet tube into the stomach.
4. Pour water into the fish's stomach via the funnel and tube.
5. Once water fills the stomach and is visible in the tube, cover the open tube end, invert the whole fish three times, and then allow water and stomach contents to empty through the tube into a container.
6. Repeat the step 5 until no additional stomach contents are collected (usually three times).
7. Use a tube as gastroscope and inspect the stomach.
8. Repeat the steps 4 to 7 until no additional stomach contents are collected or visible.
9. Place the stomach contents in the labelled plastic bag or other suitable transporting container.
10. Store the bag or container on ice, or use preservation of the sample.

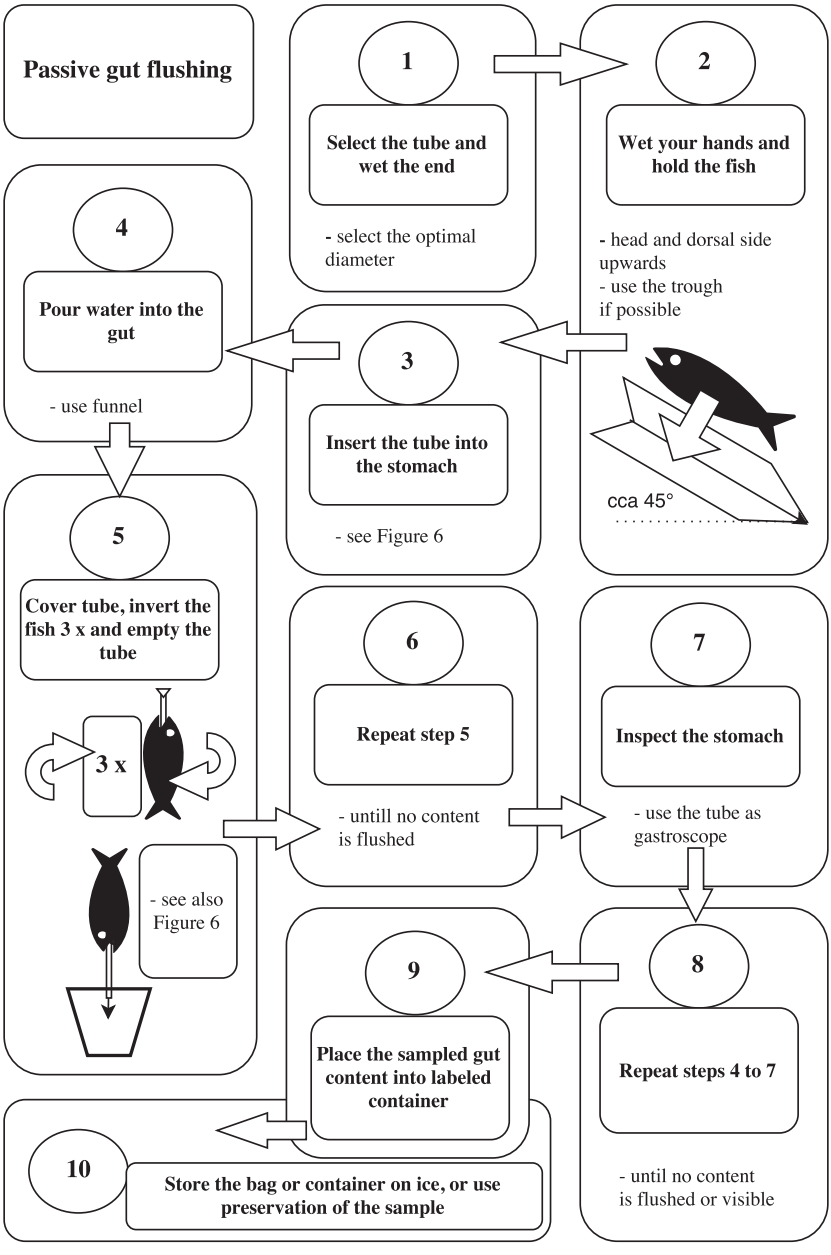

Figure 5 Schematic flowchart of the passive gut flushing.

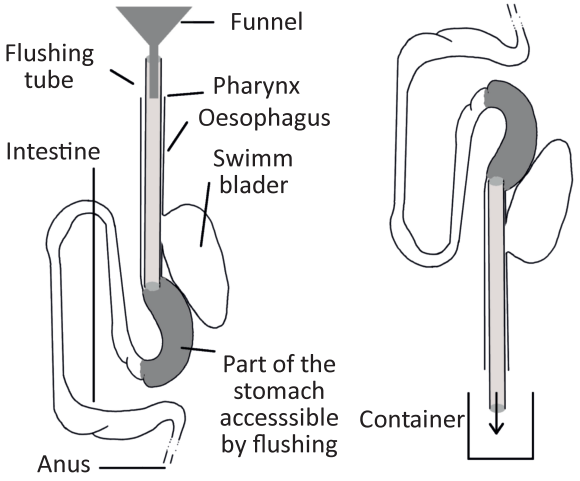

Figure 6 Placement of the flushing tube used in the gastric lavage at filling and flushing (steps 3, 4 and 5).

Active gut flushing with pumps or syringes

This method employs pulses of water to flush the stomach contents. Syringes, hand pumps, compression pumps, electrical pumps may be used to pump the water. A tube (intramedic tube) attached to the pump delivers water to the stomach cavity. Pumps are more efficient for large fish, syringes or devices with very well controlled pressure should be carefully used. A design for the stomach and anal back flushing of small fish includes a holding and collection trough, and hand syringes. Special modifications must be made for some species, thence literature review or/and pilot study on a small number of specimens are advisable (see Kamler & Pope 2001, Waters et al. 2004 and Gelwick & Mathews 2006 for more information, device description and more references).

Material: pump or syringes of appropriate capacity; intramedic tubes of different diameter; trough(s) with measure (mm scale); digital camera; labels; permanent marker; pencil; containers; clear, smooth, nonflexible tubes (plastic or glass) of various diameters with bevelled ends

Method description (Figures 7 and 8):

1. Prepare the flushing system according to the manual (or Figure 8).
2. Wet the end of the tube before inserting it into the fish. For each fish, select the largest diameter tube that easily passes the oesophagus without injury to the fish. Alternatively, select appropriate tubes for anal backwashing.
3. Using wet hands, one person holds the fish oriented with its head and dorsal side upwards in the trough.
4. Another person inserts the wet tube into the stomach. Alternatively, insert also the tube into the anus for anal backwashing.
5. Holding the tube and the fish's mouth over a trough, or container, turn on the pump/pump the water with a hand pump carefully/press syringe

carefully directing water pulses into the gut. Allow backflow to flush out the contents or monitor the backflow when using coaxial “tube in tube” system.

6. For large fish, massage the abdomen to help the efflux of water with stomach contents.
7. Repeat the steps 5 and 6 until no additional contents are flushed.
8. Use a tube (plastic or glass) of appropriate diameter as gastroscope and inspect the stomach.
9. Repeat the steps 5 to 8 until no additional stomach contents are collected or visible.
10. Place the stomach contents in the labelled plastic bag or other suitable transporting container.
11. Store the bag or container on ice, or use preservation of the sample.

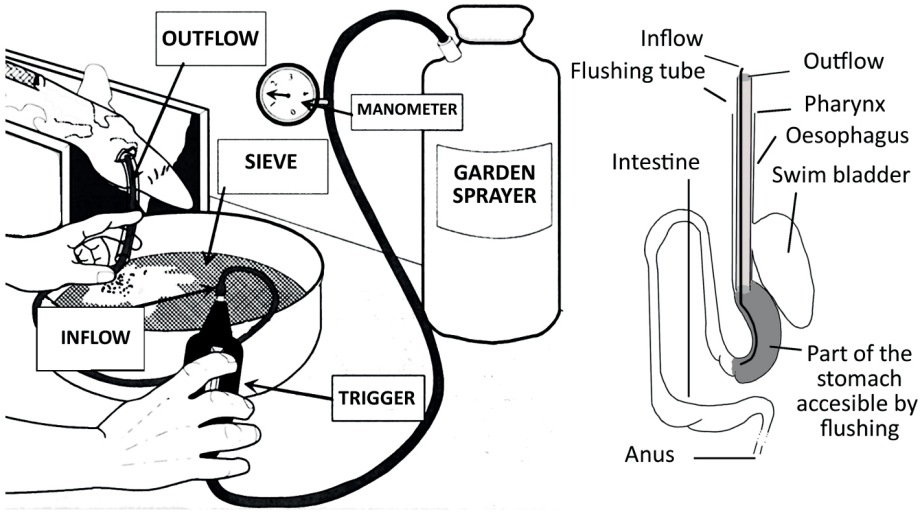

Figure 7 Active flushing using “coaxial system” and mechanical pressure. The process of flushing (redrawn according to Brosse et al. (2002)) and the tubes placement.

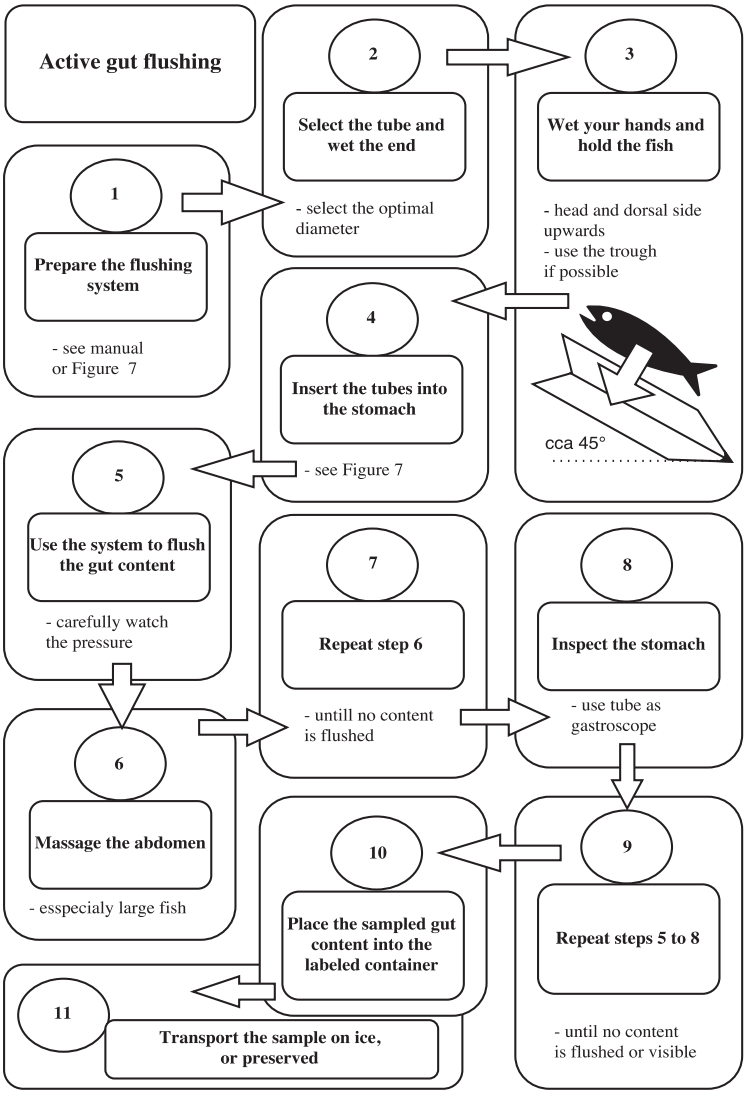

Figure 8 Schematic flowchart of the active gut flushing.

3.8 Sample analysis

Before the gut content identification (and/or quantification), it is important to record some other relevant information on the gut content. Not only the diet composition is significant, but also data on empty stomachs, fullness and the state of digestion provide very meaningful indications on the feeding habits of investigating fish. Below, we point out some reasons why to record and deal with these sample characteristics.

3.8.1 Empty stomachs – no food, no information?

It is not uncommon to find empty guts. However, investigators must be cautious about how increasing sampling effort to find fish containing food affects their estimates (Chipps & Garvey 2007). As Chipps & Garvey stated, the impact of this practice remains unexplored. Presumably, greater sample sizes arising when empty guts are frequent. It would affect variance estimates relative to other samples. Often, investigators restrict their analyses to the subset of individuals containing diet items (i.e., dropping individuals with empty guts) to explore diet preference. This practice also must be approached cautiously. Diet characteristics of fish populations for which empty stomachs were frequent may be quite different than those for which empty stomachs were rare.

In our opinion, it is always worthy to deal with fish with an empty stomach as with those with some gut contents. All the data recorded can help us analyse or explain some feeding patterns. The number of empty guts suggests the feeding activity pattern in a spatiotemporal context. More details on individuals (measure, sex, ploidy, developmental stage, etc.) with empty guts can help us to understand the intra-specific relationships and between ecotypes variations. We can use these data when analysing the feeding activity with respect to the reproductive cycles.

3.8.2 Fullness – visual assessment

The degree of fullness of the stomach should be taken into account when quantifying the gut content using some methods (Hynes 1950). This information demonstrates seasonal variation in food intake and it can be used to express between species or ecotypes variations even when the diet has identical composition. Fullness is usually estimated by considering two factors: the degree of distention of the stomach, and the mass of the bolus relative to the size of the fish. Stomach fullness may be estimated as the proportion of the maximum stomach volume occupied by prey items, or as a proportion of maximum stomach capacity. Fullness is expressed on a scale from 0% to 100%. Some other possibilities of fullness expression have been used and published, for example scale from 1 to 7 (1 – empty; 2 – trace of prey; 3 – trace-25% full; 4 – 25-50% full; 5 – 50-75% full; 6 – 75-100% full; 7 – distended; AFSC 2015), or from 1 to 4 (1 – empty; 2 – <50% filled; 3 – >50% filled; 4 – bursting; Garrido et al. 2008). Alternatively, stomach volume and the volume of each prey species may be assessed using subjective feeding units (see Knight & Margraf 1982; Pope et al. 2001; Chipps & Garvey 2007 and other references there; AFSC 2015). Because of this high number of possibilities, it should be properly identified the method used in other studies when comparing results.

Index of fullness may be also expressed by mass. This is measured as the ratio of food mass to body mass as an index of fullness, which is very widely employed. (The ratio of the corresponding volume can also be used.) This index can be applied to the food in the stomach, or to that in the whole digestive tract. It is usually expressed as parts per 10,000 (e.g., Kamler 2002; Chipps & Garvey 2007) (see also Chapters 5.4.1.2 and 5.4.2).

Some authors have also used not the actual mass (or volume) of the stomach contents, but their reconstructed mass. The index obtained has been distinguished as the index of consumption. Reconstructed weights are estimated from the lengths of relatively indigestible parts of the organisms consumed. For accuracy, it is necessary to make systematic measurements on whole specimens of various sizes, for each of the food species consumed (Pope et al. 2001; Zacharia & Abdurahiman 2004).

3.8.3 State of digestion

The state of digestion is important from more points of view. First, it indicates the accuracy and precision of the analysis. When the contents are in a higher level of digestion, it is more complicated and less precise to identify the food and even more problematic the quantification (see more in the dedicated chapters). The state of digestion also, for example, indicates when approximately the food was consumed. Differences in the state of digestion in separate parts of digestive tract are important indicators. When one food (prey) item is in the same state of digestion, the fish consumed all the content in a short period. When prey particles of the same origin are in a different state of digestion, the intervals between consuming were probably long.

An example of digestion state codes in the laboratory protocol with explanations (modified from AFSC 2015) are presented here:

1 - stomach empty

No items found in the stomach.

2 - traces of prey items

There are only a few parts left of the prey item because most of the item has been completely digested away. Use this code when you find almost completely digested prey. For example, fish bones with no flesh remaining, head capsules of chironomids or chitinised parts of arthropods with no other tissues.

3 - < 50% intact

Extensive digestion is evident but, there may be several parts and perhaps some well-digested chunks remaining. For example, squid and fish would have some flesh remaining, large crustaceans or insects may be missing parts due to digestion, and it may be impossible to distinguish individuals in a slurry of parts.

4 - 50-75% intact

Prey items are still partially intact, but remaining portions may be softened due to digestion. For example, fish would have no exposed skin remaining and parts of the head or tail may be disarticulated, but a majority of the flesh would still be present; arthropods may have most of the exoskelet and appendages intact, but have the exoskelet and internal flesh softened due to digestion.

5 - 75-100% intact

Prey items are in good to almost perfect condition, but often with some damage due to digestion. For example, fish are mostly intact with partly impaired scull consistency, but may be missing some skin or fin rays (usually the first parts of the fish to be digested away), arthropods may be missing cerci or antennae.

6 - No digestion

Prey items are in intact condition.

4 Food identification – qualitative study of the diet

Food identification is often the main aim of the gut content analysis. The qualitative analysis consists of a complete identification of the organisms in the gut contents. Only with extensive experience and with the aid of good references it is possible to identify prey and other food particles from digested, broken and fine comminuted materials.

The proper taxonomic resolution for identifying stomach contents largely depends on the research question. The level of prey identification is also determined by the researcher's skill, time available and information needed. Coarse taxonomic resolution is appropriate, for example, when quantifying ontogenetic changes in diet composition. The presence of fish in the diet may prove adequate for determining the size or the time at which fish switch to piscivory. In other instances, finer taxonomic resolution may be needed, such as determining seasonal or spatial differences in diet composition, or comparing species (Norton 1995; Gelwick & Matthews 2006; Chipps & Garvey 2007). Because food items could not be identified to species they were assigned to broader taxonomic groups, despite the researcher experience and his effort. Often it is pragmatic to reduce the number of variables (food items in this case) involved in the analysis. Sometimes, necessary pooling occurs when unidentified prey is present in the stomachs. Intuitive pooling is based on taxonomic or ecological similarities among prey (e.g., when three species with similar morphological and behavioural characteristics occur in the diet). Similarly, species representing benthic, pelagic, or littoral prey could be pooled. Finally, statistical pooling uses quantitative statistical procedures as a basis for pooling prey categories. This hypothesis that two or more prey categories act as a single resource is tested (e.g., using chi-square contingency table analysis). Positive association implies that these prey items are acting as a single resource and may be pooled (Chipps & Garvey 2007 and more references there). However, there are also reasons why to identify the diet components to lowest possible level. When the research is focused on the detailed food composition, or there may occur taxonomically close, but ecologically different food items, the lower taxonomical level can be a better solution. Also when one need to evaluate the quantity of food (prey) using calculation with size-mass regressions, different taxonomical resolution can produce differences in results (see Chapter 5.4.1.3).

It is also important to identify life history stage. One species may occur in the environment and consequently in the diet in more developmental stages. They have different nutritional value, often occur in different microhabitats and are available for different feeding strategies. Thus, all prey, even if belonging to one species (taxon) must be identified and recorded as separate food item and its stage must be recorded (e.g., egg, larva/nymph, pupa, juvenile, adult, mating pair, colony).

As an alternative to taxonomical identification, functional categories of prey can be assigned based on the apparent behavioural and functional challenges that the predator overcame in order to capture and process prey (Norton 1995).

Food items may be also classified according to the habitat in which they occur and feeding habitat preferences of the predators assessed from the frequencies of food items from different habitats (Jobling et al. 2001).

Food identification

Material: stereomicroscope and light microscope (preferably both with micrometre and digital camera); Petri dishes, entomological tweezers; millimetre paper; Pasteur pipettes; needles; water

Method description (Fig. 9):

1. Place a single sample into a Petri dish.
2. When remains of fish occur in the sample, replace them to separate Petri dish and immerse them in a porcine pancreatin solution consisting of 1 g pancreatin powder, 65 ml lukewarm tap water, and 35 ml saturated borate solution (buffer) to identify prey based on bone morphology. Measure the mass of the fish remains, place these separate and labelled subsample in a drying oven at 40°C for 2 to 24 h, depending on the size of the fish.
3. Identify prey (food) to the lowest possible (or chosen) taxon and record their presence in the laboratory form. (Treat the identified food items according to the quantitative method selected (see Chapter 5).)
4. Take photographs of items when the identification is difficult, separate the individuals and replace them into the tube with a label and preservation fluid for further identification, when necessary.
5. Count, photograph and replace parasites in a separate tube with a label and an appropriate preservation fluid (e.g., Justine et al. 2012).
6. Take note on the parasites occurrence (number, location in the digestive tract, etc.) in the laboratory form.
7. Identify the fish remains and record the presence of particular taxa to the laboratory form.
8. Repeat the step 4 when necessary.

Prey items in fish stomachs are most often not intact. Identification is much easier when the sample of accessible food is taken from the habitat and the potential food (prey) is known. A reference collection of fish hard parts, and a reference collection made on the stream site to aid prey identification, especially for benthic macroinvertebrates, may be helpful.

Otoliths or other relatively indigestible hard parts, such as scales, pharyngeal teeth, cleithra, or backbones, have diagnostic, species-specific characteristics useful for identifying fish prey (Garman 1982). Also, other food, especially arthropods are often being identified using their remains. The most chitinised parts such as head capsules, mouth parts, tarsal claws, and cases often enable to identify the prey in the order or family, rarely in the genus and in extremes in the species level, when the researcher is experienced.

Published identification keys are necessary to identify different food items. No single literature source contains keys to all the prey taxa in fish stomachs. Identification keys also deal with species of restricted region. Confirmation our identifications with a variety of more specific taxonomic references and

distribution lists is appropriate. It is also good practice to use more than one characteristic to make a positive identification, especially when identifying to the species level.

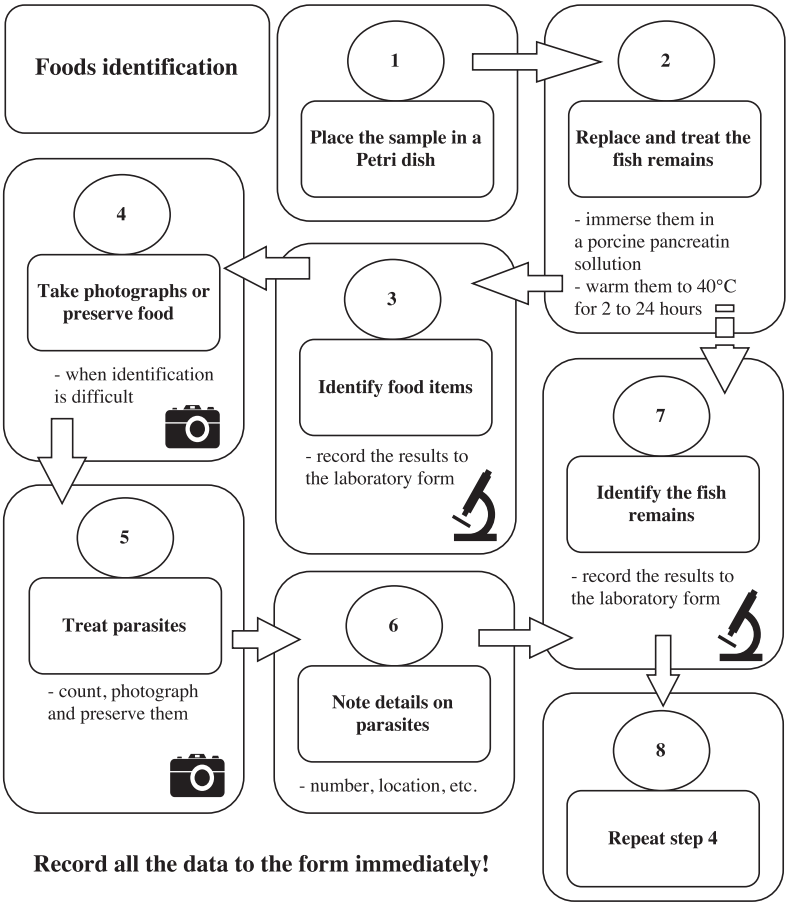

Figure 9 Schematic flowchart of the diet identification.

5 Quantitative study of the diet

Quantification of the gut content in terms of the evaluation of specific food importance is one of the most discussed problematics in the gut content analysis. Many authors reviewed existing methods, compared them, and supposed the "best" one for application in the various scenarios and for highlighting different aspects of feeding ecology (Hynes 1950; Hyslop 1980; Macdonald & Green 1983; Cortés 1997; Hansson 1998; Liao et al. 2001; Ahlbeck et al. 2012; Baker et al. 2014). Naturally, on the basis of rating and comparing food components in the diets of fish on an importance scale, one makes the assumption that some food is more important than others to the growth, survival, recruitment, size structure, condition, reproductive success, or other aspects of the ecology of the studied species. Accurately characterising the true importance of food components is thus crucial to this process (Bowen 1996). In an effort to identify dominant or important prey species researchers of animal diet have used several types of measurements. These measures were developed to fulfil a different purpose and include simple numerical and occurrence methods, methods based on biomass and energy value (Hynes 1950; Hyslop 1980; Macdonald &Green 1983). The approach which researcher choose depends not only on the purpose of the study but often on whether or not discrete items can be identified and counted. On the other side, there are many problems associated with the use of different approaches of food quantification and their importance evaluation. Deep analyses and comparisons have brought a lot of information about the advantages and disadvantages of each approach, but it is not possible to say with certainty which one is the best not only in general but often even not for specific questions of fish feeding ecology. The answer to this and the more general question of which importance index is the most accurate is complicated and has never been resolved (Liao et al. 2001). In specific studies, the choice of approach (e.g., abundance versus bulk based measures) will influence the results. An analysis of preliminary samples could help to determine the degree of shared information among the measurements (Macdonald & Green 1983).

In this chapter, we try to provide a comprehensive overview of most of the known approaches and methods critically summarizing the available information on advantages, disadvantages on the basis of published reviews and comparisons.

5.1 Frequency of occurrence, presence – absence approach (known as *F*, *%F*, *FO*, or O_i)

The presence or absence recording of each prey item across all individuals is the easiest way how to express the relative importance of various prey items and to assess the dietary composition of a fish population. The importance is inferred from the proportion of total guts containing each prey item. This traditional technique relies simply on the positive identification of some body part of the prey to provide accurate and precise data on the dietary composition (Baker et

al. 2014) and gives an indication of food item variability in the fish diets. The number of stomachs in which each food occurs is recorded and expressed as a ratio of the total number of fish examined. Often the number of occurrences of all items is summed and scaled down to a percentage basis to show the percentage composition of the diet (Hynes 1950).

Frequency of occurrence is calculated as:

$$\%\boldsymbol{F_i} = \frac{\boldsymbol{N_i}}{\boldsymbol{N}} \times \mathbf{100}$$

where ***%F*** is the frequency of occurrence of given item ***i***, $\boldsymbol{N_i}$ is the number of stomachs in which given item ***i*** occurs and ***N*** is the total number of stomachs examined.

It is easy to obtain data using this method. The only matter at issue is that the frequency of empty stomachs may change seasonally, so results may differ significantly if the frequency of occurrence is calculated on the basis of the total number of stomachs examined. This complication can be eliminated when the frequency of occurrence is calculated on the basis of the number of stomachs with food as follows:

$$\%\boldsymbol{F_{fi}} = \frac{\boldsymbol{N_{fi}}}{\boldsymbol{N_f}} \times \mathbf{100}$$

where $\boldsymbol{\%F_{fi}}$ is the frequency of occurrence of given item ***i***, $\boldsymbol{N_{fi}}$ is the number of stomachs in which given item ***i*** occurs and $\boldsymbol{N_f}$ is the total number of stomachs with some food[4].

Generally speaking, this method is easy to use, fast, it can also be executed with far less effort, and hence cost, than more detailed methods and do not require time-consuming measures or determinations. This approach at worst provides only a minor loss of information relative to more intensive and superficially detailed methods, and at best provides the only robust and interpretable models (Ahlbeck et al. 2012; Baker et al. 2014). There is a minimal risk of errors based on subjectivism when using this method. In our experience, a young inexperienced researcher can certainly assess the presence of an item in the stomach content on the basis of a small amount of retained residues of food in a short time after work under supervision.

[4] A modification of the occurrence method is the estimation of the occurrence of the dominant food in each stomach. This modification should reflect the fact, that even in opportunistic fish species, individuals are often food specialists (Amundsen et al. 1995). The occurrence of dominant food may give a quick and crude qualitative estimation of dietary composition in a population (Jobling et al. 2001). This approach was not used frequently.

This method demonstrates what organisms are being fed upon, makes possible to evaluate interspecific interactions and is also usually adequate for questions regarding the seasonal use of a prey resource (Chipps & Garwey 2007). For the descriptions of dietary composition, the frequency of occurrence provides the most robust and interpretable measure of diet composition (Baker et al. 2014). Ahlbeck et al. (2001) found out that this method also performed surprisingly well comparing estimated and true diet composition of analysed fish.

However, frequency of occurrence provides no indication of the relative importance of prey to the overall diet and has been criticised for ignoring the relative amounts of prey and giving incomplete information, since distinct food categories may be consumed with the same regularity, but in different abundance (Hyslop 1980; Kawakami & Vazzoler 1980; Bowen 1996; Lima-Junior & Goitein 2001; Chipps & Garwey 2007; Braga et al. 2012). Frost (1977) has found percent occurrence measures to be particularly appropriate only when there are few food categories. The frequency of occurrence was less robust and more variable depending on the fish species than others methods in laboratory experiments (Ahlbeck et al. 2001). Ahlbeck et al. (2001) also reported large overestimation of small prey derived frequency of occurrence both in simulations of continuously and periodically feeding fish and underestimated the diet contribution of larger prey. They argue, that particularly in piscivorous, periodically feeding fish small prey is rare and there is no digestion during feeding. Thus, small prey stays as long as large prey in the stomach. Similarly, Pierce & Boyle (1991) report exaggerating the importance of incidental prey and accumulation of food with a long passage time, resistant to digestion due to hard body parts.

5.2 Numerical abundance – the numerical method

The second traditional method, the numerical method is based on the counts of items in the gut content. The total number of individuals of each food item in each stomach are given and expressed as a percentage of the total number of food items (organisms) in all fish examined (Hynes 1950). This method has been applied successfully in studies on the food of fishes feeding on food, where the items can be counted relatively easy.

As stated above, the advantage of this method is, that it is easy in some cases. It is easy to count individuals of easily countable prey (where individuals can be easily recognised thanks to resistant body parts such like head capsules, cases, carapaxes) in the digestive tract of some fish species (e.g., insects in salmonids, small crustaceans in cyprinids). Difficulties begin when the food do not appear in discrete units (like detritus, macroalgae, pieces of plant material and plant debris), the food is masticated or fast digestible because of its nature, oligochaetes for example (Hyslop 1980; Scharf et al. 1997; Ahlbeck et al. 2001; Elosegi & Sabater 2009; Legler et al. 2010; Baker et al. 2014). Thus, the relative digestibility of prey must be also taken into account to avoid bias when both easily digested prey and resistant prey are present. Some authors suggest to sample (analyse) only the immediate foregut (i.e., stomach) where the food

remained relatively untouched and are often the only means of observing the natural diet (e.g., Williams 1981; Sutela & Huusko 2000). In the number method, the differences in size of food items are not considered, similarly to the frequency of occurrence. The special cases also are a fish species which eat, amongst other organisms, large numbers of a particularly small species. Here, the use of the numerical method can distort the results and this seems to be yet another reason why the number method should be rejected (Hynes 1950). Liao et al. (2001) and Ahlbeck et al. (2012) implicitly confirmed that this method overestimates small particles and underestimates large prey. Thus, the number method has very limited use when analysing fish with not countable food or trying to study of food items importance when food consists of significantly variable prey (food) size.

However, the number method could be applying when analysing partial differences in diet of individuals, species, ontogenetic stages of its life, bio- or eco-types, rather than expressing the value/importance of food items in our opinion, as the smallest prey sometimes contribute only little to the total prey mass but still has the same "importance" in numbers (Hynes 1950; Ahlbeck et al. 2001). When frequency method does not show any significant differences in the diet composition, there can be sometimes detected significant differences in relative amount (number, expressed as per cent) of consumed particular food. These differences may be related to slightly different microhabitat characteristics and consequently in slightly different potentially accessible food and help explain or confirm different microhabitat use as an implication of coexistence. In such situations, also partial information on the number of only some food items (or taxa, size groups of food consumed) could be useful when comparing diets and other data are not available or they are not applicable (Manko et al., unpublished data). The numerical method is informative regarding feeding behaviour (Macdonald & Green 1983) and also could reflect the effort in selecting food, but the problem of selection is very complex (see also the chapter dedicated to the food selection and electivity). It works relatively well in piscivorous and benthivorous fish species with continuous feeding and delayed sampling (Ahlbeck et al. 2001).

Summing up, this method can be helpful and useful in some special situations, when the food items (prey) can be easy and undoubtedly counted upon the understanding that results cannot be used for assessment of food items importance. Numbers of individual food items can give us important information regarding between species (biotypes, ecotypes) differences[5]. We recommend to use the numerical method very carefully in eligible situations and considering its weaknesses and limitations.

[5] When analysing differences between sites, microhabitats etc. in ecology, we also do not take the biomass or volume of organisms into account and data on the taxa numbers are sufficient. Consequently, data on food (prey) in diet are good enough for studies focused on the differences in diet of particular species, bio- or eco-types. When the differences are confirmed by this method, it is a matter for further studies to clarify the root of the difference using other suitable methods.

5.2.1 Subsampling in the numerical abundance

The food items are counted in a whole sample if it is possible and effective in terms of the ratio between effort and profit (the "return for the investment" of incremental time and effort commensurate with the improvement in data resolution). Particularly if the sample is large and relatively homogenous, researcher can choose some of the subsampling methods and analyse only a part of the total gut content. The goal of subsampling is to provide an unbiased representation of a larger sample. Subsampling must be random and should incorporate a composited field sample from several individual collections (Barbour & Gerritsen 1996). We usually homogenize samples and then sort, count, and identify a small subset of the original sample. In an attempt to both standardise and reduce collection and processing costs, a relatively small number of individuals is used to represent the assemblage. Much work has been directed toward this problematics in general ecology. Especially the taxa richness interpolating from benthic macroinvertebrates subsamples has been extensively studied because large samples with hundreds or thousands of animals in the mass of organic matter from riverbed are to be sorted. The problem is similar in some types of gut contents. There are two basic methods how to analyse subsample. They use the volume or the fixed count approach. Subsampling a fixed fraction yields an estimate of real numbers of food items in the diet of particular fish, and subsampling a fixed number of food items (prey) yields an estimate of relative abundance of food items in the gut content. The fixed number method is more efficient strategy (Walsh 1997) but it does not provide information on real numbers of prey which could be an obstruction in diet analyses. Thus, we recommend the modified method proposed by Barbour & Gerritsen (1996) in which a fixed-count approach is used in combination with randomly selected fractions or "grids" within a pan so that material and organisms from several (usually greater than 4) grids are composited to form the subsample. The size of grid depends on the mass of gut content and the size of food prevailed. Bias is minimised by requiring each fraction (grid) to be sorted in its entirety. Therefore, all food items regardless of size, colour, and morphology are sorted from the fraction. In some cases, a fraction may have to be subsampled further to prevent exceedance of the targeted number of items. The optimal target number seems to be 300 items (Vinson & Hawkins 1996). The close approximation of the targeted number of food in the subsampling is important to count effort because it is appropriate to make comparisons only between subsamples of the same (or very similar) number of organisms sorted. Subsampling should be designed to avoid exceeding the targeted number by more than 20%. Details of the subsampling problematics and more information required in some specific situations are available in many papers dealing with the subsampling of aquatic invertebrates, mainly plankton and macrozoobenthos (e.g., Van Guelpen et al. 1992; Plafkin et al. 1989; Barbour & Gerritsen 1996; Vinson & Hawkins 1996; Carter & Resh 2001; Ohman & Lavaniegos 2002; Haasse et al. 2004).

Food items counting

Material: stereomicroscope and light microscope (preferably both with micrometre and digital camera); Petri dishes; entomological tweezers; grid or millimetre paper; Pasteur pipettes; needles; water

Method description:

1. Select the target number of food items. (Best results could be obtained when selecting 300).
2. Homogenise the sample in Petri dish, ensure equitable distribution of gut content over the grids and countability (visibility) of particular items by dilution when necessary.
3. Select the appropriate scale (grid size) according to average size and density of food (Your target number should be obtained from more than four grids!).
4. Select the first grid randomly (for example use number generator to select the row and column).
5. Count all food items in the first grid.

Counting prey

If items are disarticulated or digested, a characteristic part (best if found once per prey) is counted as one food item. However, there may be ambiguity in some samples as to how many individuals of a given taxon are within the stomach. If this ambiguity exists, enter the minimum number that can be proven. Always examine and evaluate all remains of each food item. (As example: If there are 6 mayfly nymph heads and 14 left mayfly nymph legs, the prey count for mayfly nymph is 6. If 1 to 3 of those 14 legs are obviously not associated with any of the 6 heads – they are too large, or too small, the prey count is 7. This method works in carnivorous or planktivorous fish, but it does not solve the problem when fish feeds also on a carcas.)

6. If the number seems to be larger than the target number divided by five, select smaller grid size, or subsample the selected grid and repeat the steps 3 to 5.
7. Count all food items from another randomly chosen four or more grids until you reach the target number ± 20%. Record the numbers of the particular food (prey) item as $\boldsymbol{N_{sub1\ldots i}}$.
8. Record the number of grids examined as $\boldsymbol{G_e}$.
9. Count grids where gut content ***i*** occurs and record the value as $\boldsymbol{G_t}$.
10. Calculate the total numbers of a particular food items as follows:

$$N_i = N_{subi} \times \frac{G_t}{G_e}$$

where $\boldsymbol{N_i}$ is the total number of particular food item ***i***, $\boldsymbol{N_{subi}}$ is the number of particular food item ***i*** counted in selected grids (subsample), $\boldsymbol{G_t}$ is the total number of grids in which food occurs, and $\boldsymbol{G_e}$ is the number of examined grids.

11. Save $\boldsymbol{N_i}$ and $\boldsymbol{N_{subi}}$ for further analyses.

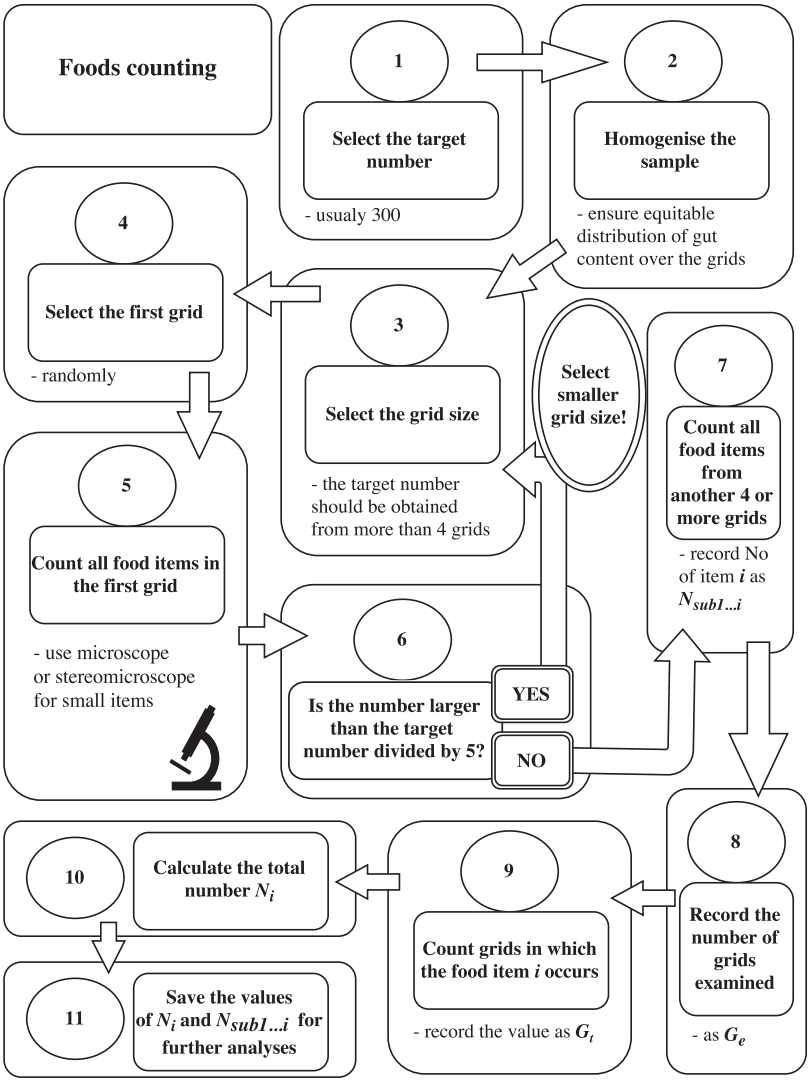

Figure 10 Schematic flowchart of the food counting with subsampling.

The objective of the diet study is to sort and identify (to chosen taxonomical level) all food items. Analyses by eye will result in fewer organisms being identified and counted than when sorting under higher magnification (Carter & Resh 2001). Sufficient magnification is, therefore, important and the grids are examined under a stereomicroscope. When working with small food particles (e.g., plankton), it is necessary to work with a microscope and use a counting chamber[6].

[6] In some cases, also visualisation of the microscopic view on the screen (pc monitor) and suitable software tools (e.g., ImageJ – see Collins 2007; Papadopulos et al. 2007; Leica Application Suite – Leica Microsystems 2006) are usable. They can help accelerate the

A haemocytometer (used for counting blood cells; Gelwick & Matthews 2006; Elosegi & Sabater 2009) or flow-cytometer works well for very small prey items.

5.3 The dominance method based on the numbers of food items according to Hynes 1950 (D_n)

The number of fish in which each food item occurs as the dominant foodstuff is expressed in one of the two ways used in the occurrence method (Hynes 1950). Essentially, the dominance method was projected as an improvement of the occurrence method. The lack of information on the quantities of the food items present in the stomach should be eliminated (Zacharia & Abdurahiman 2004), but already Hynes (1950) found out that the dominance method gives substantially the same result as the occurrence method. It also has sense only to count food occurring in discrete units (prey specimens) when the dominance is derived from numbers. Therefore, it is questionable if it makes sense to use this method in practice. If used, the dominance of particular item is calculated according to equation:

$$D_i = \frac{N_{di}}{N} \times 100$$

where D_i is the dominance of food item i, N_{di} is the number of fish in which prey of item i dominates (i.e., has the largest number) in the gut content, and N is the number of fish examined.

5.4 Methods based on the bulk (biomass)

Estimates of the fresh (wet or dry) mass (biomass) of food are often required for studies of the feeding ecology of fish. The bulk of the food items could be evaluated in many ways. Basically, there are three main ways to express the bulk - numerical, volumetric and gravimetric (Natarajan & Jhingran 1961). Another way is to use the body size - mass relationships for linear dimension-mass conversion (Benke et al. 1999; Chipps & Garvey 2007). The volume or mass (or "weight"[7]) measures reflect the dietary nutritional value of food items and also are recommended when prey (food items) are too numerous to be counted or do not occur in the diet as discrete units (Macdonald & Green 1983; Cortés 1997). The volume or mass methods are probably the most satisfactory (Hynes

process of analysis and can ensure the digital information (picture, counting outputs) stored in computer.

[7] Although weight and mass are scientifically distinct quantities, the terms are often confused with each other. In science and engineering, the weight (***W***) of an object is usually taken to be the force on the object due to gravity. The unit of measurement for weight is that of force, which in the International System of Units (SI) is the newton (N). In physics, mass (***m***) is a property of a physical body. It is a measure of an object's resistance to acceleration when a force is applied. The SI unit of mass is the gram (g). Thus, we use mass and the symbol ***m*** in this publication even if term weight and symbol ***W*** was used in original descriptions.

1950) and are usually evaluated as far the best to assess the various food items quantitatively. These methods are also a good choice when one would evaluate the energy flow (Chipps & Garvey 2007) and seem to be a suitable measure of prey importance (e.g., Grabowska & Grabowski 2005). The opinions that consider the mass-based methods as the most appropriate to express the true diet as accurately as possible are supported also by experimental laboratory study. The mass-based methods produced diets that were consistently more similar to the true diets than the other methods (Ahlbeck et al. 2012).

Of course, it is necessary to take into account also the uncertainties and weaknesses of these methods. The first and most important fact is that the separation of prey items in fish guts can rarely be carried out unambiguously. When trying to do so causes unquantifiable errors to any measure of prey bulk (Baker et al. 2014). There are several reasons why the separation cannot be provided accurately. Digestion makes the direct measurement often impossible while the prey is often not complete and the digestion rate alters between different preys (Chapter 3.8.3; Johnston & Cunjak 1999). Remains of food items are often inseparable and indeterminable. They create a mixture of digested tissues from multiple food items (Braga 1999; Lima-Junior 2000; Baker et al. 2014). Thus, the separation of food items for counting, weighing or volumetrically quantifying in an individualised way is frequently impossible and some food items cannot be allocated to any prey category with absolute confidence, regardless of how prey categories are defined (Schafer et al. 2002). The determination of fresh mass is not feasible in many cases. Consequently, samples are usually fixed soon after collection and mass is estimated from measurements of preserved prey which can potentially cause another error, direct measurement may not be because preservation often alters the mass of prey (see Chapter 3.4). Even where it is possible to accurately separate prey items in a gut or process fresh samples, the actual composition of a gut content is affected by a number of factors which cannot be expressed quantitatively (MacDonald et al. 1982; Hallfredsson et al. 2007). The mechanical prey handling, differential digestion and evacuation rates of different prey items and volumes, and the order of ingestion are reasons why bulk data contain unquantifiable error and are difficult to interpret (Hyslop 1980; Jobling 1981; MacDonald et al. 1982; Rindorf & Lewy 2004; Baker et al. 2014). It is known, that even if the skin of prey in the digestive tract may remain intact, digestion of the tissue proceeds and seemingly undigested fish taken from the gut of piscivorous species can lose a relatively high proportion of its body mass (He & Wurtsbaugh 1993). Order of ingestion and differences in digestion rates of food may lead to a great overemphasis of the importance of the prey item consumed last or digested slowest, which may be a problem when studying small sample sizes (Baker et al. 2014). Furthermore, the components that are indigestible or otherwise of limited nutritional value, molluscs shells for example (Hyslop 1980) or trichopteran cases, may have great mass and are to be eliminated from the sample before measurement which can be laborious when their abundance in the gut contents if high. The problem of bulk data interpretation is as well the unusual prey. If it occurs in the gut and is large, it may greatly influence the data obtained by the bulk measures (Salini

et al. 1990). In all cases, it is important to recognize the fundamental practical limitations of the apparently more detailed methods for quantifying gut content composition and to give careful consideration to how the detailed contents of a fish's gut, ingested in some unknown order at variable and unknown points in time, actually relates to the composition of the diet as it was ingested by the consumer (Baker et al. 2014).

5.4.1 The volumetric method

In this method, the volume of each food item, or of the total food of each fish, is given (Hynes 1950). Many workers consider the volume as a satisfactory method for quantitative analysis of gut contents. As Hynes (1950) pointed out, volume forms a very suitable means of assessment especially in the case of herbivorous and mud feeding fishes. The volume of particular food items is most often expressed as the particular food item volume percentage of the total volume of digestive tract contents. This result is obtained by using following formula:

$$\%\boldsymbol{V_i} = \frac{\boldsymbol{V_i}}{\boldsymbol{V_t}} \times \mathbf{100}$$

where $\boldsymbol{\%V_i}$ is the percentage of the item $\boldsymbol{i}$, $\boldsymbol{V_i}$ is the volume of item $\boldsymbol{i}$ and $\boldsymbol{V_t}$ is the total volume of food (gut content).

There are four possible ways how to obtain data ($\boldsymbol{V_i}$ and $\boldsymbol{V_t}$) on the volume of particular food item: (1) visual, or eye estimation method, (2) displacement volumetric method, (3) calculation using the length-mass regressions, and geometric formulas.

5.4.1.1 Visual estimation method (Eye estimation method)

Using this method, the volume of various food items in the diet is evaluated visually. The relative volume of every food item in each gut is estimated and recorded as a percentage of the total food volume directly by a researcher. This is probably the simplest and easiest means of determining the volume of food items with only little effort when comparing with others volumetric methods. However, it suffers from several weaknesses. This method of analysis is clearly highly subjective in nature and the investigator's personal bias is likely to influence the results. This flaw can be minimised by experience and training gained by examination of large samples and repeated comparison of own values evaluated in the same sample. Actually, when the method is strictly defined and rigidly enforced, it is possible to obtain relevant results with very small variance when comparing with the dry mass method. This is supported by very often used method of visual microscopic assessment of the relative density or percent composition of herbivores diet. This method was designed and tested for accuracy in terrestrial herbivores diet analysis (Sparks & Malechek 1968), but the precision of this (or similar) method should be valid also in the case of fish diet analyses (certainly at least for herbivores and detritivores). Even so, the differences

between individual investigators are generally difficult to guess and that leads to potentially incomparable results (Elosegi & Sabater 2009). This method is an alternative to the numerical method when analysing diet with food items which cannot be counted (e.g., plant material, debris)[8]. The volume can be expressed as a real ("true") volume (see step 3.a in the method description) or as a relative volume (see step 3.b). The first possibility has the advantage of comparability between samples, but there is a higher risk of inaccurate results. The second possibility has the disadvantage that does not provide the possibility to compare samples and all results give only information on a relative portion of particular food item in the diet. This method avoids the mechanical separation of food items, which could be very problematic (or impossible) in some cases. However, sometimes it is also impossible to visually separate the food items and thus, this method cannot be applied.

Volume estimation - homogeneous mass of small food items

When measuring small stomach volumes, the stomach contents are squashed on a plate to a uniform depth and the area of the squash is measured (Hyslop 1980 and more references there; Gelwick & Matthews 2006).

Material: stereomicroscope and/or light microscope (preferably both with micrometre and digital camera); Petri dishes; grid or millimetre (graph) paper; microscopic glass (or counting chamber, or microscopic glass with squared index)

Method description (Fig. 11):

1. Material must be flattened to a uniform known thickness (e.g., 1mm) on a Petri dish, or microscopic glass (or microscopic glass with squared index, or counting chambers) when very small amount and size of food items. (You can use pieces of microscopic glasses or counting chambers with a known thickness to ensure the accurate thickness of analysed sample.)
2. This step can be performed several ways (a-c), according to available/ preferred equipment:
   a. Place the dish over graph (millimetre) paper. Estimate the area covered for each prey item by counting grid cells which are covered by particular item ***i*** under a stereomicroscope with appropriate magnification.
   b. Use square indexed reticula in a stereomicroscope. Estimate the area covered for each prey item by counting grid cells which are covered by particular item ***i*** under stereomicroscope or light microscope with appropriate magnification.
   c. Use a microscopic glass with squared index. Estimate the area covered for each prey item by counting grid cells which are covered

[8] An alternative approach to calculation of the food item mass is to use appropriate software (e.g., Collins 2007; Papadopulos et al. 2007; Leica Microsystems 2006). It is possible and more accurate to rely on computer technology when estimating biomass of small food particles than try to estimate the mass of small food items such like plankton by visual assessing (but also calculate using geometric equations, weighting or displacement). It is possible to frame manually the areas which are covered by specific food item, use semiautomatic, or fully automatic functions, modules or plug-ins to obtain data on relative portion of different food items in the gut content.

by particular item ***i*** under stereomicroscope or light microscope with appropriate magnification.

3. This step can be performed two ways (a, b), depending on required results (absolute and relative (a) or only relative (b) values):
   a. Convert area to true volume using formula

$$\mathbf{V}_i = N_{ci} \times a^2 \times h$$

   where V_i is the volume of item ***i***, N_{ci} is the number of grid cells covered by food item ***i***, a^2 is the area of one cell (***a*** is the length of one side of square), and ***h*** is the thickness (height) of the flattened mass of gut content expressed in the same unit as ***a***.
   b. Convert area to the relative volume of particular food item using formula

$$\mathbf{V}_i = N_{ci} \times a^2$$

   where V_i is the volume of item ***i***, N_{ci} is the number of grid cells covered by food item ***i***, and a^2 is the area of one cell (***a*** is the length of one side of square).
4. Calculate the volume for each prey this way and the total volume of gut content as a sum of particular food volumes.
5. Calculate the relative volume of each food item as a percentage using the generalised equation in Chapter 5.4.1.

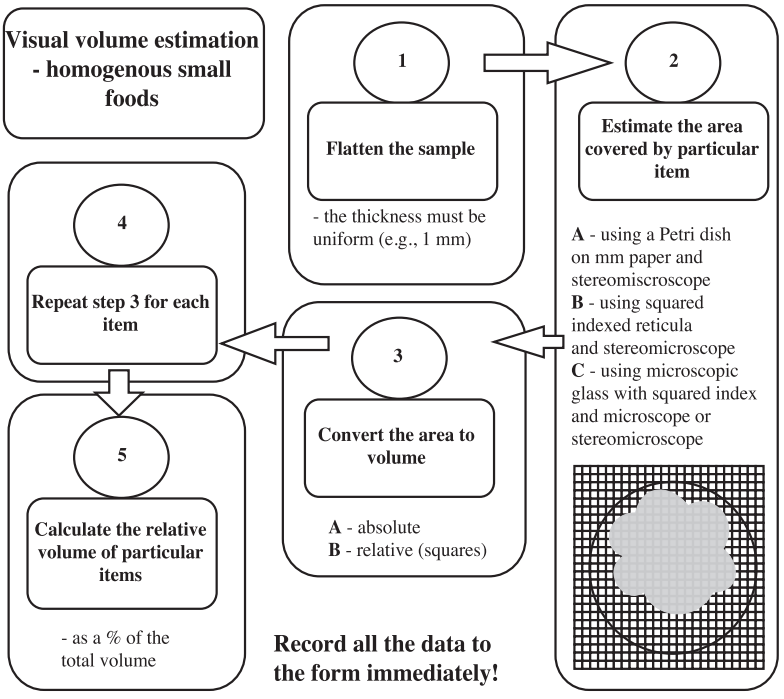

Figure 11 Schematic flowchart of the visual volume estimation (homogeneous mass of small food items).

5.4.1.2 Displacement method

The volume of each food item, or of the total food of each fish, is given and is usually expressed as a percentage of the total mass (weight) of the fish (Hynes 1950). The displacement volume method uses measurement in a calibrated graduated cylinder (Turingan 1994). This direct method is probably the most accurate one for assessing the volume. The volume of each food item is measured by displacement in a graduated container such as a cylinder with the smallest possible diameter for accuracy and could be used for calculation of these ratios (Hynes 1950). The displaced volume is equal to that of the food item. Alternatively, the "settled" volume of the stomach contents may be measured by allowing them to settle in a graduated measuring vessel (Hyslop 1980). This method is eminently suited in the estimation of the food of carnivorous fishes eating larger items (prey) rather than for small and/or rare occurring food (which can often have the volume smaller than the divisions on graduated cylinders scales, even if the cylinder has smallest possible diameter, such as small crustaceans, algae, diatoms, etc. in small plankton or detritus feeders). Although it is possible to measure volume of small prey or food not occurring in discrete units such as algae and detritus using the displacement method when these food items occur in larger masses as well as they can be separated from each other, there are other limitations the importance of which increases with decreasing

dimension of food items. A major problem with direct estimates of volume based on displacement is that the water trapped within the food may cause large errors in the estimate. Excess water can be removed by blotting items on filter paper before volume determinations are attempted, but, especially in the case of small items, this water is often difficult to extract (Hyslop 1980). Another limitation of this method is the differential rate of digestion of the food items. It may significantly affect the accuracy of the observations. However, if the collections are made when the fish are on feed, this issue can be overcome. A knowledge of the volumes of the different size groups of the food items may be of great help in indirect estimating of the volume if the whole item is created by semi-digested fragments (Zacharia & Abdurahiman 2004). But, other problems are connected with this solution, because laborious measurements of prey are necessary to assess the volume accurately. As the food items may change their volume differently in preservation media (see Chapter 3.4), the evaluation of the portion of various food can also vary from the real situation. This fact makes also a comparison between authors or studies less reliable. Another one relevant objection may be the presence of large volumes of mucus in some species could make this method more difficult (Hynes 1950; Baker et al. 2014).

Displacement method – measurement of large food items volumes

There are several variants of this method. We describe two (A and B) most common of them.

A. The volume measurement is based on the immersion of the food item into the water in a calibrated graduated cylinder. The difference between the initially known volume of water and final volume express the volume of the food item.

Material: calibrated graduated cylinder; distilled water; tweezers

Method description (Fig. 12):

1. Prepare a calibrated graduated cylinder with as small diameter as possible for a particular food item. The cylinder should have a large capacity, small scale grades and high precision of measurement (small deviation).
2. Fill the cylinder to known volume which will ensure that the food will immerse completely but the volume will not overreach the scale. Record the volume as $\boldsymbol{V_w}$. Filling water to the dedicated value, control the water level with eye accurately on the same level.
3. Separate the food item ***i*** from the other gut contents and remove the excess water touching the tissue paper.
4. Place the food item into the cylinder.
5. Measure the volume and record the value as $\boldsymbol{V_t}$ with eye accurately on the same level.
6. Calculate the volume $\boldsymbol{V_i}$ of item ***i*** using formula:

$$\boldsymbol{V_i = V_t - V_w}$$

7. Calculate the volume for each prey this way and the total volume of gut content as a sum of particular food volumes.
8. Calculate the relative volume of each food item as a percentage using the generalised equation in Chapter 5.4.1.

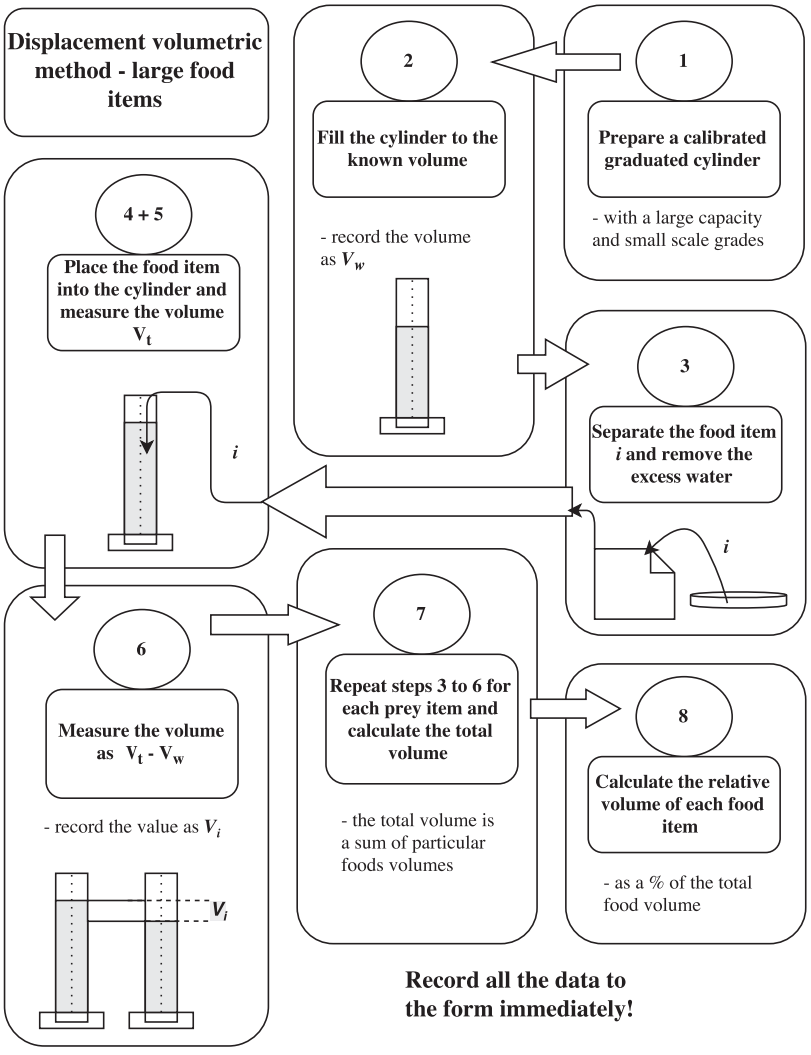

Figure 12 Schematic flowchart of the displacement volumetric method (large food items).

B. The volume measurement is based on the immersion of the food item into the water in a calibrated graduated cylinder. The difference between initially known volume of water and final volume expresses the volume of the food item.

Material: two calibrated graduated cylinders; distilled water; tweezers
Method description:

1. Prepare a calibrated graduated cylinder (I.) with as small diameter as possible for the particular food item. The cylinder should have a large capacity, small scale grades and high precision of measurement (small deviation).
2. Prepare second cylinder (II.) and fill it with distilled water to known volume and record it as V_a.
3. Separate the food item from the other gut contents and remove the excess water touching the tissue paper.
4. Place the food item into the cylinder I.
5. Fill the cylinder I. with water from the cylinder II. to dedicated volume V_c so that the food item is immersed completely.
6. Measure and record the resultant value in cylinder II. as V_b.
7. Calculate the volume V_i of item ***i*** using formula:

$$V_i = V_c - (V_a - V_b)$$

8. Calculate the volume for each prey this way and the total volume of gut content as a sum of particular food volumes.
9. Calculate the relative volume of each food item as a percentage using the generalised equation in Chapter 5.4.1.

Index of fullness expressed by volume – The mean stomach fullness index

This index is calculated as the ratio of observed prey volume to estimated stomach capacity (Kimball & Helm 1971; Knight & Margraf 1982). The total volume of prey in each stomach is estimated either directly by water displacement or indirectly by means of geometric measurements. Maximum total prey volume is then regressed against fish size to estimate maximum stomach volume as

$$V_s = a \times L^b$$

where V_s is maximum stomach capacity, a is regression coefficient, L is total length, and b is the instantaneous rate of change (Knight & Margraf 1982; Pope et al. 2001; Gosh et al. 2009). The ratio of observed prey volume (V_i) to maximum stomach volume (V_s) provides an index of stomach fullness that accounts for fish length and is calculated as

$$MSF_i = \frac{1}{P} \sum_{j=1}^{P} \left(\frac{V_{ij}}{V_{sj}} \right)$$

where MSF_i is the mean stomach fullness index of food ***i***, P is the number of fish with food in their stomachs, ***j*** is fish, V_{ij} is the volume of the food category ***i*** in fish ***j***, and V_{sj} is the stomach capacity of fish ***j*** (adapted from Pope et al. 2001).

The ***MFS*** as the stomach fullness index is then calculated as a sum of particular values obtained for individual food items (but it is naturally possible to calculate the index directly with the loss of information on particular food).

This index has several advantages when comparing with other fullness indexes: (1) it eliminates subjectivity associated with the points method, (2) it is relatively quick and easy to apply, (3) it can be obtained from both preserved or live fish, and (4) it can be analysed by a variety of statistical procedures. It also correlates well with prey caloric contribution, providing a robust index for evaluating the energetic contribution of different prey items (Knight & Margraf 1982; Pope et al. 2001). This index also expresses the absolute and relative portion of individual food categories, which makes it usable not only as the fullness index but also a tool for evaluating the diet composition and importance of particular components.

The dominance method based on the mass of food

When it has no sense to count food not occurring in discrete units, it is possible to use volume instead of numerical abundance. The bulk of the food material is taken into account. This method may be an extension of other method based on bulk (mass). After measuring (estimating) the relative volume or mass of feeding items, the fish in which particular food item dominates are counted. The dominance method based on the volume is similar to the dominance method according to Hynes (1950), which is based on numerical abundance. Advantages and disadvantages of these approaches result from the advantages and disadvantages of numerical and mass-based groups of methods in general.

5.4.2 Gravimetric method

Total prey mass can be measured by subtraction of empty gut mass ("weight") from total gut mass before dissection. The mass of each food item, or of the total food of each fish, is given and is usually expressed as a percentage of the total mass of the fish (Hynes 1950). The advantages and disadvantages of the gravimetric method are similar to the displacement method. Stomach mass content may be expressed as wet, dry or ash-free dry mass (Hyslop 1980). Generally, the wet mass of the food is measured after removing extent water by blotting with tissue paper to minimalize the bias caused by measuring food items with water trapped between the food pieces. Another way to elude this issue is to measure the dry mass of food in the gut content. Dry mass estimation is more time consuming and is usually employed where accurate determinations of calorific intake are required. Dried items can be weighted if they are large enough to be handled individually and have been digested only slightly (Bowen 1996). Dry mass is determined after drying to constant mass (usually by oven-drying at 60 - 105°C for 48 hours). When very accurate results are needed, samples are cooled down in a vacuum desiccator and then weighed. Because detritus can make up a large part of some diets, stomach contents can be combusted and the ash-free dry mass (AFDM) calculated as dry mass minus ash mass. Most

prey items can be considered 100% organic matter for these calculations, but it is necessary to adjust the mass of molluscs for shell mass (Gelwick & Matthews 2006). Ash-free dry mass involves ashing samples in a muffle oven (furnace) at 450–550°C, cooled down in a vacuum desiccator and weighed to obtain the ash free dry mass. Mass-specific stomach content may be calculated as milligrams dry mass of food per gram fresh mass of fish. As an alternative to bulk weighing, each prey item may be weighed. This may be possible for large prey, but small zooplankton may be difficult to sort and weigh individually (Hyslop 1980; Jobling et al. 2001). The limitation of mass as a criterion of analysis is still current because microbalances for routine weightings to 10^{-7} g (e.g., for small crustacean dry mass measurement) are, until today, not common equipment in ecological laboratories. Besides these, the accurate weighing of small quantities of food matter is extremely difficult and impracticable in studies of large collections.

Some authors (e.g., Ricker 1937; Neill 1938) calculated the mass of food eaten from the known average mass of each individual of each food item, index of fullness based on the mass of the gut content was developed (see below) and even more variations of this method were used in fish gut content analyses. According to the aim of study, quantity, diversity and digestion degree of the gut contents, a researcher can opt the most suitable variant or a combination of variants when using the gravimetric method. In any case, the formalin preservation may cause an increase in mass (as we pointed in Chapter 3.4) and other preservation liquids can cause mass loss of prey particularly of those with larger body size. For this reason, it is not suitable to use gravimetric methods of diet analysis in preserved samples.

Bellow, we describe the procedure of weighing on the example of wet mass. The procedure of dry mass variation is similar but requires more steps to obtain dry sample or ash-free dry mass. As there are numerous variations of the basic methodology, researchers can modify or/and add individual steps to the procedure. These steps are generally described in the text above and their application depends on the equipment of the laboratory, research focus, properties and composition of the analysed stomach content. Two of many possible alternatives are shortly outlined as footnotes.

Mass measurement – wet mass

Material: entomological tweezers; Pasteur pipettes; tissue paper; Petri dishes; precise analytical balance

Method description (Fig. 13):

1. Reset the balance with Petri dish to zero.
2. Separate the least presented food item to the Petri dish used in step 1.[9, 10]

[9] Alternative 1 - Large food items, little or no damage by digestion: the process can be carried out the way that each individual of larger prey or particular piece of larger food and all types of non-food remains are processed separately. Then, the sum of mass of all particular individuals/pieces is counted as a wet mass of the food item. For example, use this procedure when there are fishes or large invertebrates (nymphs, molluscs) in the gut.

[10] Alternative 2 – Numerous individuals/pieces of food items, little or no damage by digestion: when the gut content is relatively extensive, there are only few food items left after less numerous food have been sorted out and digestion has done little damage, the mass is determined as

3. Blot the extend water with tissue paper.
4. Measure the mass of the food item ***i*** and record the value as m_i.
5. Repeat steps 1 to 4 until all identifiable food items are weighted.
6. Reset the balance with Petri dish to zero.
7. Separate the unidentifiable food items to Petri dish used in step 6.
8. Measure the mass of the unidentifiable food item and record the value as m_u.
9. Reset the balance with Petri dish to zero.
10. Separate remaining non-food items (stomach lining, parasites, or rocks, etc.)
    to Petri dish used in step 9.
11. Measure the mass of remaining non-food items and record the value as m_r.
12. Sum the values of particular food items $m_{i1...n}$ and the mass of the unidentifiable food m_u to obtain the total mass m_t (wet mass) of the digestive tract content of analysed fish.
13. Calculate the relative proportion of each food item as a percentage using following formula:

$$\%m_i = \frac{m_i}{m_t} \times 100$$

where $\%m_i$ is the percentage of item ***i***, m_i is the mass of item ***i*** and m_t is the total mass of food (gut content).

14. Calculate the relative volume of unidentifiable food items as a percentage using following formula:

$$\%m_u = \frac{m_u}{m_t} \times 100$$

where $\%m_u$ is the percentage of the unidentifiable items ***u***, m_u is the mass of the unidentifiable items and m_t is the total mass of food (gut content).

15. Calculate the relative volume of non-food remains as a percentage using following formula:

$$\%m_r = \frac{m_r}{m_t + m_r} \times 100$$

where $\%m_r$ is the percentage of non-food remains ***r***, m_r is the mass of non-food remains and m_t is the total mass of food (gut content).

---

follows: The remainder of the sample is rinsed and placed in a quartering dish. In one quadrant of the dish, all food items are separated and removed. Then, each food item is weighted and multiplied by 4. For example, use this procedure when there are many copepods, amphipods etc. in the gut content.

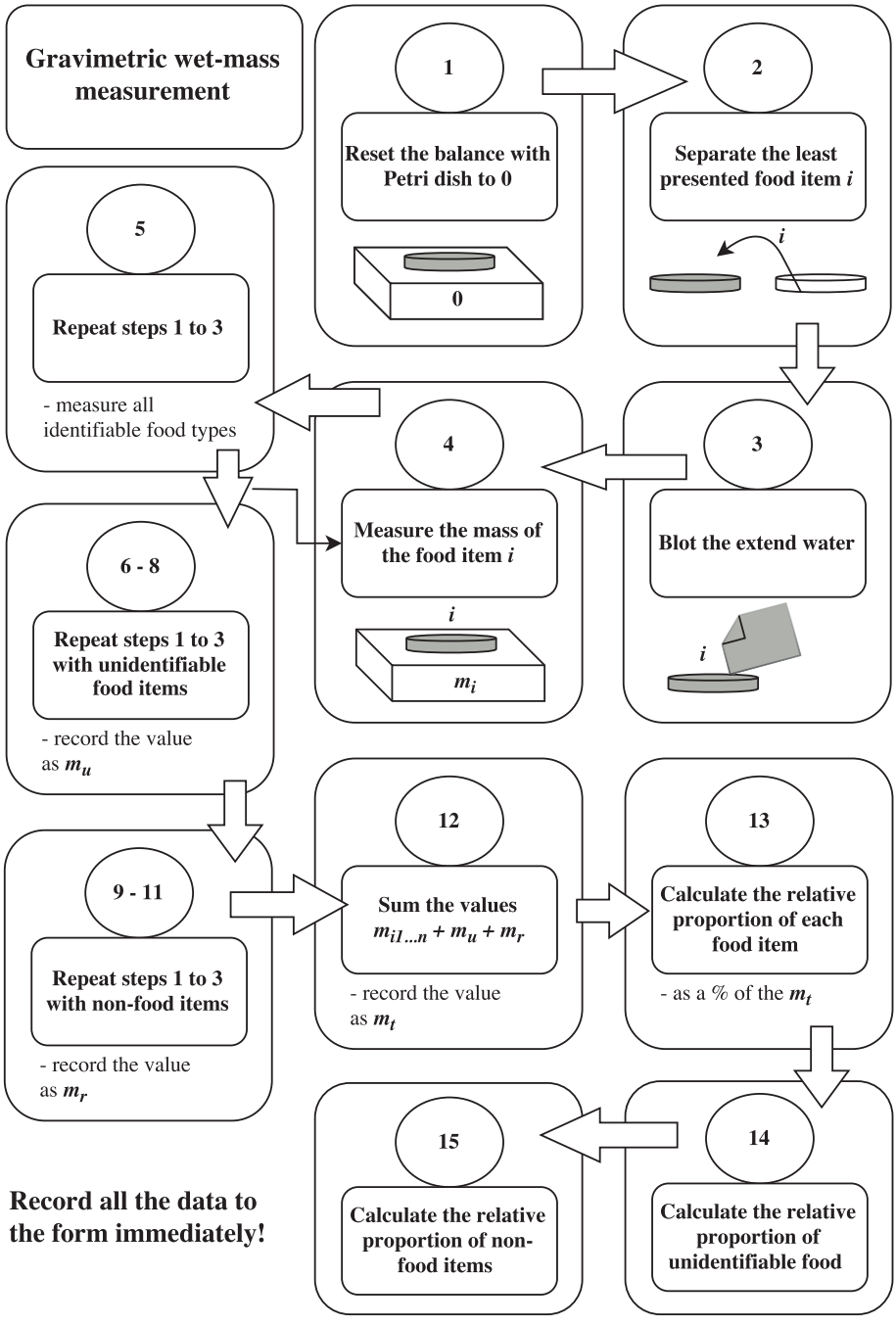

Figure 13 Schematic flowchart of the wet mass measurement.

Index of fullness expressed by mass (ISF)

This index expresses the ratio of food mass to body mass. It is very widely employed and can be applied to the food in the stomach, or to that in the whole digestive tract. It is usually expressed as parts per 10,000 (‰) and calculated using formula:

$$ISF = \frac{m_g \times 1000}{m_s}$$

where ***ISF*** is the Index of fullness, m_g is the mass of the gut content mass (g) and m_s is eviscerated body mass (g) (e.g., Kamler 2002).

5.4.3 Calculation of volume and mass

The relationship between body mass and relatively easy measurable body proportions such like length or head width is a useful tool in ecological research (e.g., Culver et al. 1985; Langeland et al. 1991; Kawabata & Urabe 1998; Miyasaka et al. 2007) and can be effectively used also in the study of fish diet. When the prey item is too small to be weighed accurately, when also measuring of the volume is impossible or impractical from the same or from another reason, it is possible to indirectly calculate biovolume or mass of some types of food. Accordingly, the main reasons for their use are: (1) they allow avoidance of biases caused by the mass losses of preserved animals, (2) they make possible further work with the stored samples, and (3) they can be less time-consuming and more precise than direct weighing or biovolume estimation in works requiring measures of all the specimens (prey, food) in the samples (González et al. 2002, and many references there).

Length-dry mass regressions are the most widely used approach for estimating benthic invertebrate biomass because they are faster and more precise than other methods (Burgherr & Meyer 1997; Benke et al. 1999). They allow estimation of prey biomass in a predator's gut (particularly equations for head width) even when the prey may be torn apart or partially digested (Benke et al. 1999; Karlson et al. 2007). Hard and relatively indigestible parts of the organisms consumed (for example shells, chitin, bones, otoliths, scales or carapace) are often used to determine lengths or mass of prey items by regressing the dimension of an indigestible hard part against whole-body length or mass (Chipps & Garwey 2007). It is even possible to reconstruct size and mass of crushed bivalves and amphipods from the taxonomically relevant parts of the exoskeletons in the intestinal mucus hulls (Brandner et al. 2013).

We describe a technique of aquatic macroinvertebrates mass calculating as an example of often used approach bellow.

Length-mass method – calculating the biomass as a dry mass in aquatic invertebrates

I. Linear regression development and calculation of the dry mass

Non-preserved (fresh) animals collected for this purpose at the sampling station provide the best results since preservation (especially in ethanol) results

in shrinkage of soft body parts and losses of dry mass by leaching. Animals preserved in a formalin solution will provide estimates comparable to non-preserved specimens (see also Chapter 3.4).

Material: entomological tweezers; stereomicroscope and/or light microscope; preferably both with micrometre so that lengths can be measured to at least 0.1 mm (or use digital camera and measurement software); microscopic glasses; Petri dishes; precise analytical balance

Method description (Fig. 14):

1. Select at least 20 specimens of prey ***i*** from a wide range of size categories.
2. Measure the head width as the heavily sclerotised head capsule is more resistant to damage than other insect body parts of individual animals under a microscope or stereomicroscope and record the measures as L_i for each animal. (See publications and manuals dedicated to microscopic measuring to follow the convenient procedure for specific method and equipment!)
3. Place the measured individuals in separate Petri dishes with labels which will allow assigning dry mass to the particular individual.
4. Dry the measured individuals in a drying oven for a minimum of 24 h at 60°C.
5. Cool down the measured individuals in a desiccator.
6. Weigh the measured individuals on an analytical balance with acceptable precision and record the values as m_i.
7. Develop the regression. Use the function ***regression*** and ***power*** (or parabolic) ***curve*** in a statistical program or spreadsheet processor to obtain the regression of the formula

$$m_i = a_i \times L_i^{\,b_i}$$

where m_i is the mass of item ***i***, L_i is the length of particular item ***i***, a_i is the constant and b_i is the slope of regression, both specific for the item ***i***. Use manual for chosen program or processor, if necessary. Alternatively, you can also use the formula

$$\ln m_i = \ln a_i + b_i \times \ln L_i$$

This equation is the linear equivalent of a power curve. Since we expect a cubic relationship between L and W, b should be reasonably close to 3 (Benke et al. 1999). The third alternative is the formula

$$\log m_i = \log a_i + b_i \times \log L_i + e$$

where the e is a normally distributed error term with mean 0 and variance equal to the residual mean square of the regression (Bird & Prairie 1985).

8. Separate individual prey items from the digestive tract into different Petri dishes.
9. Measure the head width of each individual of one prey item (taxon) and record the values as L.

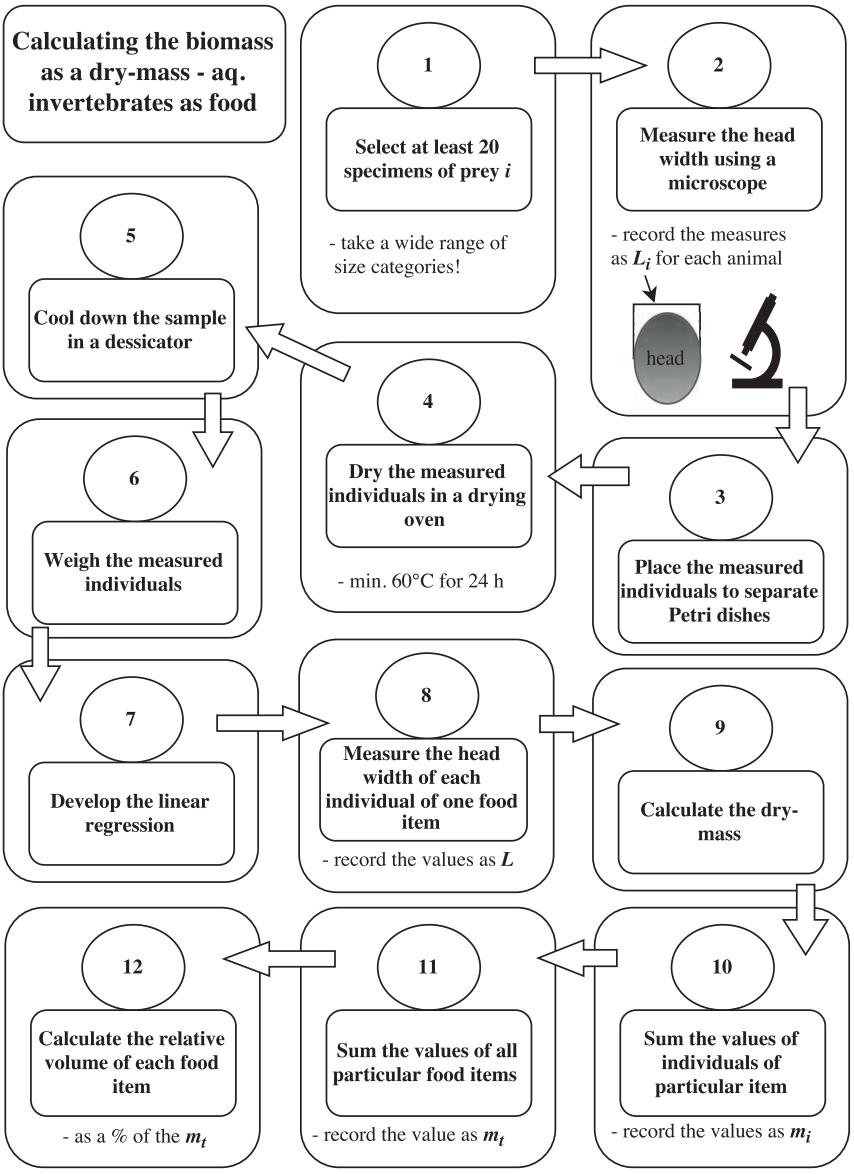

Figure 14 Schematic flowchart of the dry mass measurement (aquatic invertebrates as food).

10. Calculate the dry mass of each prey (individual) backwards using formula from step 7 (in spreadsheet processor to computerise the process).
11. Sum the values of individuals to obtain the dry mass of the particular food item $\boldsymbol{m}_i$ (e.g., prey species).

12. Sum the values of all particular food items to obtain the total mass $\boldsymbol{m_t}$ (dry mass) of the digestive tract content of analysed fish.
13. Calculate the relative volume of each food item as a percentage using following formula:

$$\%\boldsymbol{m_i} = \frac{\boldsymbol{m_i}}{\boldsymbol{m_t}} \times \mathbf{100}$$

where $\boldsymbol{\%m_i}$ is the percentage of item ***i***, $\boldsymbol{m_i}$ is the mass of item ***i*** and $\boldsymbol{m_t}$ is the total mass of food (gut content).

II. Calculation of the dry mass using published data

It is implicitly possible to use already published values ($\boldsymbol{a_i}$ and $\boldsymbol{b_i}$) to obtain length-specific mass. Some authors reviewed and summarised these data for large geographical areas, for instance Benke et al. (1999) for North America, Johnston & Cunjack (1999) for North-Eastern America; Beer-Stiller & Zwick (1995), Burgherr & Meyer (1997) for streams in Europe; Towers et al. (1994) for invertebrates from New Zealand. Many other papers on the length-mass relationships of particular species, taxa groups and ecological groups were published for freshwater invertebrates and can be used in diet analyses (e.g., Cianciara 1980; Schoener 1980; Smock 1980; Cressa & Lewis 1984; Wenzel et al. 1990; Cressa 1998; Whiles et al. 1999; Poepperl 2000; González et al. 2001, 2002; Baumgärtner & Rothhaupt 2003; Stoffels et al. 2003; Genkai-Kato & Miyasaka 2007; Miyasaka et al. 2008; Martins et al. 2014). Also for other taxa which often create a significant part of the fish gut content were published usable data (e.g.; Kawabata & Urabe 1998; Rosati et al. 2012 for marine invertebrates, and McCauley 1984; Culver et al. 1985; Lawrence et al. 1987; Meyer 1989 for freshwater zooplankton).

Some issues may be related to this second solution (using published data). The difficulty is the level in which the prey is determined and taxonomic level for which the regressions are published. Commonly, the published data generalise the regressions for genera, sometimes also for the family level. The potentially biasing effects of intraspecific differences are probably often underappreciated. For example, the study of Stoffels et al. (2003) documents high variation in parameter values among published length-mass models for the family Chironomidae. Application of models developed for higher taxa levels may result in a high degree of error in predicted mass/body length, as the parameters can vary significantly among genera and species according to the results of Stoffels et al. (2003). This is the result of many morphologically distinct genera and species (Johnston & Cunjak 1999) in this large family. On the other hand, Méthod et al. (2012) argue that much greater error can probably arise because of methodological or regional and latitudinal differences and considered the results provided by family level equations are probably valid. Some authors report intraspecific variation in size–mass relationships for stream invertebrates (e.g., Benke et al. 1999; Johnston & Cunjak 1999). The variability in parameter values for the same taxa (sometimes species) among studies may

be caused by either natural spatial and/or temporal variability in invertebrate morphology caused by temperature, chemistry, and food availability, but also by methodological differences during model development (Short et al. 1987; Griffith et al. 1993; Basset & Glazier 1995; Johnston & Cunjak 1999; González et al. 2002; Stoffels et al. 2003; Méthod et al. 2012). Therefore, caution is often suggested when using regressions performed in different regions and higher taxonomic level. Another problem can be that the back-calculated estimates may compound error in estimates of total prey mass (or volume) when biometric relationships and measurements of hard parts used to reconstruct diet items are not precise and biased (Chipps & Garvey 2007). An issue may be also the fact, that some authors do not use head width, but body length for regression development. As it is impossible to rely on the body length of prey in the gut content, the conversion between body length and head width is necessary. It brings additional possible inaccuracy and error to the calculation and makes this technique less accurate.

Material: entomological tweezers; stereomicroscope and/or light microscope; preferably both with micrometre so that lengths can be measured to at least 0.1 mm (or use digital camera and measurement software); microscopic glasses; Petri dishes

Method description:

1. Separate individual prey items from the digestive tract into different Petri dishes.
2. Measure the head width of each individual of one prey item (or another body measure according to method which was used for development of the regression formula in publication used) and record the values as ***L***. (See publications and manuals dedicated to microscopic measuring to follow the convenient procedure for specific method and equipment!)
3. Calculate the dry mass of each prey (individual) backwards using the formula from the publication which is used (in spreadsheet processor to computerise the process).
4. Sum the values of individuals to obtain the dry mass of the particular food item $\boldsymbol{m_i}$ (e.g., prey species).
5. Sum the values of particular food items to obtain the total mass $\boldsymbol{m_t}$ (dry mass) of the digestive tract content of analysed fish.
6. Calculate the relative volume of each food item as a percentage using following formula:

$$\%\boldsymbol{m_i} = \frac{\boldsymbol{m_i}}{\boldsymbol{m_t}} \times \mathbf{100}$$

where $\boldsymbol{\%m_i}$ is the percentage of item ***i***, $\boldsymbol{m_i}$ is the mass of item ***i*** and $\boldsymbol{m_t}$ is the total mass of food (gut content).

There are also many other equations scattered in works on growth and production of fauna which can be used this or similar way and other approaches which are used less often. For example, indirect volumetric analysis can be done by comparing food items with blocks of known volume. Calculation of the mean

dimensions of prey species, based on measurement of a number of individuals, allows determination of mean volume. The formula employed depends on which three-dimensional shape the organism most closely resembles (Hyslop 1980). Another method was evolved (Ricker 1937 in Hyslop 1980), in which the mass is stated by counting and "weighting" each food item organism according to its known average mass. In food items with relatively simple shapes like phytoplankton, the volume can be calculated by laborious linear measurements, shape estimation, modelling the volume equations and calculating the volume. For some groups also existing models can be used (e.g., Sun & Liu 2003)[11]. A size reconstruction of fish prey in the gut content may be possible using the size of some non-digestible parts (Jobling et al. 2001) such like the otoliths (e.g., Ogden 1970; Hernández-García 1995; L'Abée-Lund et al. 1996), or vertebrae (e.g., Damsgård & Langeland 1994).

5.5 Points volumetric method

The points method is a variation of the eye estimation method and should also be an improvement on the numerical method where consideration is given to the bulk of the food items. It should avoid the information loss this way. Each food item in the stomach is allocated points in proportion to its visually estimated contribution to gut volume (Hyslop 1980). There are more variants of this method according to different authors, some of them take into account both the size of the fish and the fullness of the stomach. This method is quite useful for analysing omnivorous and herbivores where measuring volumes of microscopic organisms such as diatoms and filamentous algae are very difficult (Zacharia & Abdurahiman 2004). Although the volume or mass methods are probably the most satisfactory, the points method is a shortcut to the same result (Hynes 1950). Also, Ahlbeck et al. (2012) found this method easy and fast. It could be preferable, as they will allow for more fish to be examined and it performs well according to their results. The points method allows estimation of the volume of each prey category without the need to physically separate the gut contents. It is a significant advantage when the food items do not occur in the form of discrete units. In the other hand, some authors have criticised this method and appoint its disadvantages. As an example, the separation must be carried out visually, and even under simulated ideal conditions using discrete artificial prey items, estimates of composition using the points method are highly subjective (Marrero & Lopez-Rojas 1995; Baker et al. 2014). Hyslop (1980) also criticised the points method for being too subjective in his review of methods. Hynes (1950) correctly notes the fact, that the results given by the points method cannot be used for comparison with counts of the organisms in the habitat. Another issue is that a possible distortion of results may occur, especially when fish of distinct sizes are grouped in one sample (Lima-Junior & Goitein 2001). From the variety of methods based on points allocation, we generally describe the method

[11] Researcher who planes to use these, or other existing methods of biomass calculation is obliged to study dedicated literature resources since it is not possible to describe all these rarely used methods in this publication.

suggested by Hynes (1950) to explain the principle. This method is simple and was improved many times. These attempts have led to very complicated and difficult techniques (e.g., the method suggested by Lima-Junior & Goitein 2001).

The points method according to Hynes (1950)

This method is rapid and easy, requires no special apparatus for Measurement, is not influenced by the frequent occurrence of small organisms in small numbers, nor of heavy bodies, like snail shells and caddis cases, and does not involve trying to count large numbers of small and broken organisms. It also does not give the spurious impression of accuracy which is given by some other methods (Hynes 1950).

Material: entomological tweezers; stereomicroscope and/or light microscope; microscopic glasses; Petri dishes

Method description:

1. Prepare the sample on Petri dish (as described in steps 1 to 14 in Chapter 3.6.2).
2. Identify present food items.
3. Allot the points to the stomach content in the first instance. In allotting the points, the size of the fish and the fullness of the stomach are also taken into account. A full stomach, irrespective of the size of the fish, is receiving a total of about 20 points, and a distended stomach receiving about 30. Record the value as $\boldsymbol{P_t}$.
4. Subdivide the points allotted to the stomach to each food item. Give always only 1, 2, 4, 8 or 16 points, and no intermediate values. Subdivide the points from step 3 to each food item according to the volume of it present in the stomach. In this way, one large organism counts as much as several small ones. Record the point as $\boldsymbol{P_i}$.
5. Calculate the relative abundance of particular food items in the investigated digestive tract. Sum all the points gained by each food item and scale down to percentages, to give percentage composition of the food of all the fish examined as follows:

$$\%\boldsymbol{P_i} = \frac{\boldsymbol{P_i}}{\boldsymbol{P_t}} \times \mathbf{100}$$

where $\%\boldsymbol{P_i}$ is the percent contribution of item $\boldsymbol{i}$, $\boldsymbol{P_i}$ is the value of points allotted to the item $\boldsymbol{i}$, and $\boldsymbol{P_t}$ is the number of points allotted to the stomach.

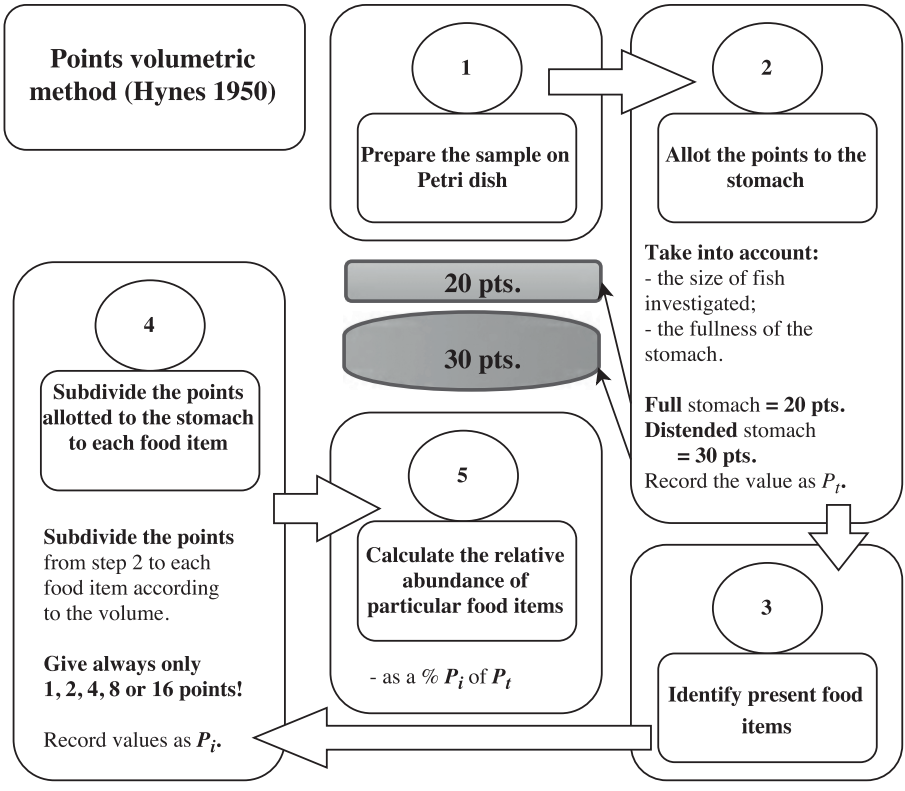

Figure 15 Schematic flowchart of the Hynes' point method.

6 Evaluating the importance of specific food using compound indexes

Stomach content can give us data which helps us to answer more complex questions of fish ecology. To make the best account of the data on the gut content, we have to evaluate the importance of particular kinds of food in the fish diet. The importance is invaluable information providing in-depth insight into the fish feeding ecology, resources demands and resources availability, potential competition and other aspects of fish ecology and biology growth, consumption or predation (Liao et al. 2001). In praxis, accurately quantifying importance of food itemss and understanding the contributions of various food to consumer or predator well-being is essential for effective management of fisheries resources (Bowen 1996) and for qualified protection decisions and actions (Pusey & Arthington 2003; Hoggarth et al. 2005). Using methods described in previous chapters, data on diet composition are obtained. They also often offer the possibility to express relative portion of individual food item in the diet and some authors use the percentage by number (***%N***), mass (***%m***), volume (***%V***), and occurrence (***%O***) to express the relative importance of prey taxa. Among them, ***%m***[12] (or ***%V***) has been the most popular index to describe prey importance and its relationships with fish well-being and prey availability (e.g., Wahl & Stein 1993; Hartman & Brandt 1995; Persson & Hansson 1999). However, in other's view, these information do not always indicate the real importance of particular food (e.g., from the nutritional value point of view). Thus, many researchers developed a variety of other methods evaluating the importance of food. We present some of them below.

Specific diet measures provide unique information about relative importance of particular food. In an attempt to receive more complex and objective information, and to avoid information loss, compound indexes were developed. They combine two or more diet measures into a single index. The authors who developed these indexes believe, that compound indexes as more advanced analytical methods capture more information than do single component measures and are more representative (Cortés 1997; Chipps & Garvey 2007; Gelwick & Matthews 2006). In addition, compound indexes should compensate the biases associated with different classic methods (e.g., Christensen 1978; Cortés 1997). Liao et al. (2001) believe that a compound index of prey importance should contain a balance of information on the contribution of prey taxa to nutrition of the predator population as a whole and the likelihood of taxa occurring in the diets of individual predators. Their results indicate that compound index (specifically %IRI) fulfills these requirements and is the optimal mix of importance characteristics for many studies wishing to convey a general notion of the importance of various prey taxa. In the other hand, these authors hereby further acknowledge that compound index (%IRI) cannot avoid all of

[12] ***%W*** in original papers.

problems and there may be situations (such as similar size among dominant prey taxa) in which use of a single component index may be preferable.

Several authors, however, find the compound indexes quite inconvenient. They often argue that compound indexes provide little or no additional information than that provided by single indexes (MacDonald & Green 1983; Rosecchi & Nouaze 1987; Hansson 1998). In addition, some authors argue, that compound indexes are difficult to interpret and analyse statistically (Rosecchi & Nouaze 1987), the addition or multiplication of percentages has no biological meaning because both quantities are dimensionless ratios (Bowen 1996). Moreover, compound indexes can be affected by the taxonomic resolution of prey items (Hansson 1998; Cortes 1997) and they possibly multiply the underlying biases and unquantifiable errors inherent in the individual parameters included in these indexes which increase the number of error sources (Hyslop 1980; Baker et al. 2014). Ahlbeck et al. (2012) confirmed by experiments that compound index (specifically ***%IRI***) produces results which significantly deviate from the true diet.

As presented, the usefulness of compound indexes is constrained by several limitations, they can be a redundant source of information and thus, it may not be necessary to combine all measures into one index especially if one measure describes most of the information (MacDonald & Green 1983).

6.1 Index of relative importance (*IRI*)

One widely used in fish diet studies is the index of relative importance (e.g., Pinkas et al. 1971). When calculating ***IRI***, the percent frequency of occurrence of each prey category (***%F***) is multiplied by the sum of the percent by volume (***%V***), or mass (***%m***)[13] and percent by number (***%N***). ***IRI*** is a composite index used to characterize diets and identify the relative importance of common food categories (Pinkas et al. 1971; Prince 1975). The three standard dietary measures are used to compute the ***IRI*** as follows:

$$\boldsymbol{IRI_i} = (\%\boldsymbol{N_i} + \%\boldsymbol{V_i}) \times \%\boldsymbol{F_i}$$

or alternatively

$$\boldsymbol{IRI_i} = (\%\boldsymbol{N_i} + \%\boldsymbol{m_i}) \times \%\boldsymbol{F_i}$$

where $\boldsymbol{IRI_i}$ is the index of relative importance of the food item ***i***, $\boldsymbol{\%N_i}$ is the percentage of specific food category by number, $\boldsymbol{\%V_i}$ is the percentage by volume, $\boldsymbol{\%m_i}$ is the percentage by mass[14] and $\boldsymbol{\%F_i}$ is the frequency of occurrence.

[13] Even the percentage of points were used (e.g., in George et al. 2009).

[14] Some authors use also percent of total dry mass of stomach content (e.g., Atkinson & Percy 1991).

Index of relative importance is one of most used compound indexes but it is also one of most criticised and given as an example when in appointments of the compound indexes deficiencies (see Chapter 6 and the literature cited therein). It is a typical controversy when, despite ample evidence of irregularities, authors rely on results obtained using this index, because they probably believe that there is no better option or do not believe that simpler way (component indexes) could be better and will give more robust and easier interpretable results.

6.2 Percent index of relative importance (%*IRI*)

The comparisons among food items using ***IRI*** values are difficult, because they are not expressed in percent. It is, therefore, suggested (e.g., Cortés et al. 1996; Desmond et al. 2002), to express ***IRI*** on a percent basis, such that ***%IRI*** for a specific food category ***i*** (***IRI***$_i$) is calculated using total ***IRI*** value summed across all prey items as follows:

$$\%IRI_i = 100 \times \frac{IRI_i}{\sum_{i=1}^{n} IRI_i}$$

where ***%IRI***$_i$ is the Percent index of relative importance of food item ***i***, ***IRI***$_i$ is the index of relative importance of food item ***i*** (Chapter 6.1) and ***n*** is the total number of food categories considered at a given taxonomic level.

The %IRI also suffers from important shortcomings of compound index criticised in more papers and reviews (see Chapter 6). On the other side, Liao et al. (2001) believe that this index is the optimal mix of importance characteristics for many studies wishing to convey a general notion of the importance of various prey taxa. They argue that balanced importance ratings and minimal size bias observed in their comparisons of %IRI with other indexes support this conclusion.

6.3 Modified percent index of relative importance (%*MIRI*)

Liao et al. (2001) applied the ***%MIRI*** (Pitcher 1980, 1981) in empirical comparison with commonly used component indexes and ***%IRI***. This index is calculated using formula:

$$\%MIRI_i = 100 \times \frac{\%F_i \times \%m_i}{\sum_{i=1}^{n}(\%F_i \times \%m_i)}$$

where ***%MIRI***$_i$ is modified percent index of relative importance of given item ***i***, ***%F***$_i$ is frequency of occurrence of given item ***i*** and ***%m***$_i$ is the percentage of item ***i***.

In the study of Liao et al. (2001), the correlation strength of ***%MIRI*** with ***%m*** was very high (higher than the correlation with ***%IRI***). Although ***%MIRI*** was originally developed for overcoming the disparity between small and large prey, results of this study suggest that it responds very similarly to ***%m*** and

emphasises the importance of large prey taxa. These facts make the relevance of ***%MIRI*** very dubious and, in our opinion, this index has actually no support for its use in practice.

6.4 Other compound indexes

Some authors used other compound indexes combining different single component indexes or combining the same indexes different way. We present here four of them as examples even if we consider them not more appropriate or for use in comparison to the indexes presented in previous chapters.

Hobson (1974) developed **Ranking index (*RI*)** which is calculated using formula:

$$\boldsymbol{RI_i = \left(\%F_{fi}\right) \times \frac{V_{si}}{V_{st}} \times 100}$$

where RI_i is the Ranking index of the item ***i***, $\%F_{fi}$ is the frequency of occurrence of given item ***i***, V_{si} is the volumetric scale of prey item ***i***, is the volumetric scale of prey items combined.

The volumetric scale value of each prey item is the result of the point method also developed by Hobson (1974). Each prey item is scaled from 0 to 1 using a 0.05 by point method, with the total contents regarded as 1 in this point method.

The **Relative importance index (*RII*)** (George & Hadley 1979) is a linear combination of three single measures:

$$\boldsymbol{RII_i = 100 \times \frac{\%F_i + \%N_i + \%m_i}{\sum_{i=1}^{p}(\%F_i + \%N_i + \%m_i)}}$$

where RII_i is the Relative importance index of the item ***i***, $\%F_{fi}$ is the frequency of occurrence, $\%N_i$ is the proportion by number and $\%m_i$ is the percentage of the mass of given item ***i***.

As stated for probably all compound indexes, it is unlikely that the ***RII*** is more accurate than a single index from more reasons. First, it is confounded by two sources of error and variation (error associated with percentages and error associated with frequency of occurrence) (Hyslop 1980); second, arithmetic manipulation of percentages, which are dimensionless ratios, produces numbers of no interpretable meaning (Bowen 1996); and third, the ***RII*** index produces a single value with no variation for each prey taxon consumed by a population (thus, no straightforward statistical comparisons of ***RII*** values can be made among populations, life-history periods, etc.) (Pope et al. 2001).

In pursuit of perfection in terms of the food importance, also the bioenergetics approach and caloric content of prey have been taken into account. As example,

the **Prey importance index (*PII*)** developed by Probst et al. (1984) combines information on the abundance, mass, and caloric content of prey:

$$PII_i = \frac{1}{N_f} \times \sum_{j=1}^{N_f} \left(\frac{m_{ij} \times X_i}{\sum_{j=1}^{Q} m_{ij} \times X_i} \right)$$

where PII_i is the Prey importance index, N_f is the total number of stomachs with food, m_{ij} is the mass of prey item ***i*** in fish ***j***, X_{ij} is the caloric density ($J.g^{-1}$ of wet mass) of food item ***i***, and ***Q*** is the number of food items (adapted from Pope et al. 2001).

The usefulness of a caloric-based index (such as the ***PII***) is that it provides a quantitative measure of the nutritional benefit of individual prey rather than relative importance based on numbers, mass, or occurrence in the diet (Chipps & Garvey 2007). The ***PII*** does not account for seasonal differences in total caloric intake; however, seasonal comparisons of ***PII*** values can be made (Pope et al. 2001).

The **Index of preponderance (*IP*)** developed by Natarajan & Jhingran (1961) produces a single value for each attribute based on frequency of occurrence and mass using the equation:

$$IP_i = 100 \times \frac{V_i \times F_i}{\sum_{i=1}^{p} V_i \times F_i}$$

where IP_i is the Index of preponderance, $\%V_i$ is the percentage of the volume of item ***i***[15], $\%F_i$ is the frequency of occurrence of given item ***i*** (adapted from Natarajan & Jhingran 1961).

A comparison of the values obtained enables a ranking of the prey in order of mathematical dominance as an expression of the importance within the diet and authors of this index are convinced it has immense advantages especially when studying fish diet in open waters where animals have access to diverse organisms (Mohan & Sankaran 1988), who also consider it to be an objective and suitable measure of prey dominance within the diet. On the other side, this technique does not distinguish between the importance of prey items by mass or occurrence and it is not suitable for dietary comparisons (Marshall & Elliot 1997).

[15] Some authors (e.g., Marshall & Elliot 1997) also use percentage of mass (*%**m***) *of* item ***i.***

7 Selectivity and preference of food items

Even if we can say what food item, what prey is important for the specific species, population, bio- or eco-type, the very fact does not automatically mean that the feeder selects or prefers this food. We can only say that resources (food item in our case) are to be selective when they are used disproportionately to their availability. The availability of resources is not uniform in nature, and use may change as availability changes. Therefore, used resources should be compared to available (or unused) resources in order to reach valid conclusions concerning resource selection (Manly et al. 2002). It is often assumed that a species will select resources that are best able to satisfy its life requirements, and that high-quality resources will be selected more than low-quality ones. Determining which food items are selected more often than others is of particular interest. It provides fundamental information about the nature of fish and how they meet their requirements for survival (Manly et al. 2002). On the other hand, differential resource selection is one of the principal relationships which permit species to coexist (Rosenzweig 1981). To use data on the diet composition obtained by the gut content analysis is, therefore, the next higher level of their utilisation and appreciation.

It is obviously clear that the evaluation of quantitative diet composition is extremely complicated problematics. When we try to assess the selectivity or preferences in feeding, we have to deal with another at least as the difficult question: What does "available" food mean? Also, the difference between selection and preference is often not distinguished. Even terms use, selection and preference have been applied interchangeably when discussing food use patterns, resulting in some confusion (Litvaitis 2000; Manly et al. 2002). First, therefore, let's define the terms. According to Johnson (1980) and Manly et al. (2002), the ***usage*** of a resource is defined as that quantity of the resource that is utilised by an animal (or population of animals) in a fixed period of time. The ***availability*** of a resource is the quantity accessible to the animal (or population of animals) during that same period of time. Accessibility is very important (as explained below) and it makes the difference to the ***abundance*** which is the quantity of that component in the environment (accessible plus not accessible). ***Selection*** is the process in which an animal chooses a resource among alternative food that is available. ***Preference*** is independent of availability. It is the likelihood that a resource will be selected if offered on an equal basis with others (cafeteria experiment)[16]. We prefer to do not use the term "preference" in this publication and understand the "preference" as stated above: as a specific case of selectivity in special circumstances when studied food item is offered on an equal basis with others, which is implicitly possible particularly in experimental conditions.

[16] Notice the contradiction ("independent of availability" *versus* "offered on an equal basis") that contributes to misunderstanding. Some authors have recognised the difficulty and rather stay out of using term "preference", but others have termed a component "preferred" if its usage exceeded its availability, and "avoided" if the reverse was true.

7.1 Available food resources

As indicated, any evaluation of the selection of food can be accomplished only with information on food availability. Unfortunately, the availability of forage or prey species can be difficult to estimate. The difficulties result from more reasons. Estimates of relative abundance are often expressed in units that do not necessarily correlate with real abundance (density) or biomass. In addition, abundance (or biomass) is often used as a surrogate to availability without considering the limitations of estimates of availability (Litvaitis 2000)[17]. What we quantify as food availability may be quite different than what is really accessible food for fish in natural conditions. To estimate available food resource, in fact, means to obtain an unbiased sample from habitat accurately representing the relative abundances of potential food items as it is encountered by the consumer (Strauss 1979). A plenty of publications deal with potential problems with the accuracy of benthic samples (e.g., Resh 1979). Using samples of prey abundance to estimate their availability for predators means in practice to sample accurately that samples reflect relative prey densities, predators encounter prey at rates corresponding to measured prey density and the predator perception of available prey is the same as that of the investigator (Peckarsky 2006). Nevertheless, the definition of availability of food for specific species, population, or even size group, bio- or eco-type of population depends on the investigator. It is improbable that any researcher can see the resources as a fish, thus, he makes this decision somewhat arbitrarily (Kohler & Ney 1982). This dependency (= subjectivity) is a more important factor as may first appear (Johnson 1980)[18]. First, it may be hard and complicated to determine the suitable scale for assessing the availability. Should researcher assess the availability of particular food components at the study site, or in habitat in which studied species occurs, or even only in the microhabitats where this species feeds? All of these possibilities have many pros and cons. Only the fact, that fish occurs at some site (or in some habitat) may itself indicate that the fish has already made a selection and its presence may suggest that it selected that site in part because of the food items available there (Litvaitis 2000). The habitat (or microhabitat) selection may also be influenced by the predator occurrence and thus, food

[17] Furthermore, because different food may occur in different (micro) habitats, a single sampling technique may not adequately quantify the relative abundance of different food items in the environment (Chipps & Garvey 2007). It brings other methodological annoyances into this problematics and investigator should master variety of different sampling methods to have the opportunity to choose the optimal combination for particular study.

[18] Johnson (1980) describes a nice example which illustrate the point: Suppose an investigator collects a fish, and finds that its stomach contains food items A, B and C in some percentages. A sample of the animal's feeding site at the time the fish was collected reveals that the items were present in some proportions. Many investigators would conclude that Item A is avoided, because usage was less than availability, while Items B and C are preferred, because usage exceeded availability. But suppose another investigator, equally familiar with the biology of the fish, does not believe that Item A is a valid food item (perhaps he thinks it is ingested only accidentally while the animal is consuming other food). He would then consider the data, obtained by deleting Item A from the analysis. Now, the assessment of Item B has changed from preferred to avoided (shortened from Johnson 1980).

which is potentially available turns into inaccessible (e.g., Gilliam & Fraser 1987; McIvor & Odum 1988; Hugie & Dill 1994). That can change in periodical rhythm or irregular according to the circadian (or others) cycles and other, perhaps accidental impacts which often cannot be taken into account. Thence, it is important to decide upon the timing, subpopulation, and activity to address in studies focused on food availability and selection because pooling information across times, subpopulations or activities may result in erroneous inferences (Manly et al. 2002). Social interactions and competition are other factors that may affect the availability and accessibility of particular food for individual fish or part of the fish population (Perry & Pianka 1997) and it is hence important to regard sex, age class and potential competitors. These facts and assumptions question the reliability of the accuracy and correctness of the real food selection assessment. Thus, the researcher should consistently consider all important facts and questions when assessing the availability of food resources in a partial study. His conclusions about selectivity are critically dependent upon the array of components the investigator deems available to the animal (Johnson 1980). In any case, he should be able to justify the statement and the methodological approach which leads to the decision on what is or is not available and accessible food resource. It must be always respected that the right way is to sense, explain and interpret results of food availability assessment (and thus food selection) only in relation to specific circumstances and as a flexible variable under different conditions. Unfortunately, it seems so that despite the effort, it is impossible to obtain an adequate estimate of the availability of food resources in some conditions (e.g., small consumer, small size of the food particles, and muddiness of the habitat) (Hynes 1950).

7.2 Selection of food resources

Differential resource selection is one of the principal relationships which permit species to coexist (Rosenzweig 1981). When resources are used disproportionately to their availability, use is said to be selective (Johnson 1980; Litvaitis 200; Manly et al. 2002). It is often assumed that a species will select resources that are best able to satisfy its life requirements, and that high-quality resources will be selected more than low-quality ones. However, there are included much more factors and variables and this research area is much more complex and complicated than it first looks. Factors contributing to resource selection include population density, competition with other species, natural selection, the chemical composition or texture of forage, heredity, predation, habitat patch size, inter-patch distances and it is also affected by season, sex, age class, behavioural activity, and daily activity pattern of the fish studied (Manly et al. 2002). Numerous models and theories of resource selection have been proposed that incorporate subsets of these factors. In an effort to understand it, ecologists have organised their evaluations of food selection into so called optimal foraging theory (Pyke 1984; Perry & Pianka 1997)[19] and many foraging models (see Manly et al. 2002 for more references).

[19] It is not possible to explain this theory here, but researcher can only profit when he is familiar with it. He can better understand and interpret the diet selection as the consequence of

To estimate selectivity from field data, investigators compare the relative importance of each prey item in the predator's gut contents to its relative abundance in the habitat. Basically, there are two approaches of comparison. The first one is the simplest method of correlation which involves comparing the ranks of prey items in the predator's guts and in the habitat using Spearman's rank correlation analysis (Siegel 1956). A significant positive correlation indicates no selectivity (similar ranks of prey items in the diet and in the environment). No correlation or significant negative correlations suggest selective predation (feeding is disproportionate to the availability of prey in the environment) (Peckarsky 2006). The other way involves calculations of various electivity indexes. Some of them are presented in next chapters.

7.2.1 Indexes of food selectivity

Early researchers simply described their findings on food use and availability. Then, some early studies indicated the number of animals consuming each prey item and the percentage of consumption. Variability among animals and locations made difficult for researchers to compare their results because differences were assessed subjectively until Scott (1920), as the first author to quantify selection, divided the average number of each prey species per fish stomach per unit of time by the number found in plankton hauls per unit area. This first index used the ratio of the rate of consumption of a prey item to the density at which it was present (Manly et al. 2002). Then, numerous other indexes have been proposed, from which we below describe the most often used ones[20].

*7.2.1.1 Forage ratio (***FR***; Selection ratio; Preference index)*

The forage ratio developed by Savage (1931) uses the relative quantity (percentage) of food item ***i*** in the gut as a proportion (percentage) of the total gut content and the relative quantity of the same food item in the environment as a proportion (percentage) of the total abundance of accessible food in the environment. The forage ratio is calculated using formula:

$$FR_i = \frac{r_i}{p_i}$$

where FR_i is the forage ratio, r_i is the relative quantity (portion, percentage) of the food item ***i*** in the digestive tract content, and p_i is the relative quantity of the food item ***i*** in the environment[21].

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fairly complex interactions of external (prey availability, risk of predation, social interactions, competition), internal (animal condition or hunger, learned experiences, age, sex and reproductive state, macro- and micronutrient requirements, concentration of toxins or distasteful compounds), and phylogenetic (morphological constraints such like mouth shape, sensory limitations, physiological limitations) factors.

[20] Some of the number of other indexes which could be theoretically used in the fish feeding ecology are also the ***Importance*** according to Bowyer & Bleich (1984), ***Selection intensity for continuous data*** **(SI)** by Rondorff et al. (1990), ***T*** by Durbin (1998) or ***Z*** by Tokeshi & Daud (2011).

[21] The value of p_i is calculated as , where u_i is the value of food units of the food item ***i***, and u_t is the total number of used food units. The value of r_i is calculated as , where a_i is value of available food units of food item ***i***, and a_t is the total value of available food units in environment.

The forage ratio shows a value of 1.0 for random feeding and changes asymmetrically when usage differs from availability. Values from 0.1 to 0.99 mean avoidance and values higher than 1.0 (1.1 - ∞) indicate positive selection of the food item ***i***[22]. This index was used relatively often only in early studies and it has some limitations and weaknesses according to several authors. The weak spot of this index is difficult evaluation in practice because this index is open-ended, suffers from asymmetry and it is sensitive to sampling error for rare or little-utilised food. It is also inappropriate to quantitatively compare ***FR*** obtained from different samples because it reflects selection for the particular circumstance observed (Strauss 1979; Lechowicz 1982; Manly et al. 2002).

*7.2.1.2 Ivlev's index of electivity (*E*; Index of selection; Ivlev's Forage Ratio)*

The Electivity index according to Ivlev (1961) is still widely used in comparing the feeding habits of fishes. The Ivlev's electivity index uses the relative abundance of prey item ***i*** in the gut as a proportion (percentage) of the total gut content and the relative abundance of the same prey item in the environment as a proportion (percentage) of the total abundance of available prey in the environment. This index was developed to characterise the electivity as a degree of selection of particular prey species by predator studied. Ivlev (1961) developed electivity index to avoid the weakness of ***FR*** resulting from the 0 to infinity range. The Ivlev's electivity index has possible range from -1 to +1. Negative values are interpreted as avoidance (or sometimes inaccessibility) of available food, zero means random selection from the environment and positive values indicate active selection. Ivlev's index of electivity is calculated as follows:

$$E_i = \frac{r_i - p_i}{r_i + p_i}$$

where E_i is the Ivlev's index of electivity, r_i is the relative quantity (portion, percentage) of the food item ***i*** in the digestive tract content, and p_i is the relative quantity of the food item ***i*** in the environment.

First assumptions that this index is unbiased and relatively independent of sampling size were later, after empirical and theoretical re-evaluations declared as invalid by Strauss (1979). He confirmed that this index is (similarly to ***FR***) significantly biased when the size samples from the gut and from the environment are unequal, it is dependent upon sample size (both relative and absolute) and is also not useful for prey not dominant in the environment. This weak point will influence the results concerning rare food items no matter how large the samples are (Lechowicz 1982). Another problem is the extreme values (-1 and +1). The -1 value (total avoidance) can be obtained only in case when the food item does not occur in the digestive tract, but occurs in the environment

The value of food unit means the value of mass, volume or numerical abundance. The selection of the quantification method depends on the researcher's decision. (Criteria for selection, advantages and disadvantages of individual methods are described in the Chapter 5.)

[22] This undesirable characteristics of ***FR*** can be avoided by taking the log ***FR*** as the index (Jacobs 1974; Cock 1978).

regardless how scarce (e.g., one individual in sample with thousands of others food items or preys) or abundant it is. In opposite, the maximal positive selection can be obtained only in the case when the food item or prey do not occur in the environment, but occurs in the gut content, no matters in how large or small is its proportion (Straus 1979). Researchers must be aware of potential misleading of these results and interpret experimental data accordingly. This index also reflects selection for the particular circumstance observed (Pearre 1982) and does not estimate any biologically meaningful value (Manly et al. 2002).

In practical terms, this index can be useful and reliable for planktivores, but not for predators of larger and less abundant prey. It is also possible to compare quantitatively the selection of particular food items obtained from different samples when the relative abundance of this food item (prey) in the environment is the same. Other ways, it is only allowable to compare results of electivities within multispecies samples using rank order comparison.

*7.2.1.3 Jacob's modified forage ratio (***log Q***; Jacob's first selection index)*

The modification of the forage ratio was proposed and highly preferred by Jacobs (1974). This modified version of forage ratio was developed based directly on the rates of decrement (mortality) of the food due to feeding, and Jacobs (1974) promised it as independent of the relative abundance. This index is calculated using formula:

$$log\ Q_i = \frac{r_i \times (1 - p_i)}{p_i \times (1 - r_i)}$$

where $log\ Q_i$ is the Jacob's modified forage ratio, r_i is the relative quantity (portion, percentage) of the food item ***i*** in the digestive tract content, and p_i is the relative quantity of the food item ***i*** in the environment.

The modification of ***FR*** has the same advantages and disadvantages as ***D*** (see below). It has a range from plus to minus infinity, but maximal values of preference (+ ∞) and avoidance (- ∞) can be obtained only in case with two food items. This index reflects selection for the particular circumstance observed and it does do not estimate any biologically meaningful value (Manly et al. 2002). It is also unusually sensitive to sampling error when availability (abundance) or utilisation are less than about 0.1, and thus has little practical value (Lechowicz 1982).

7.2.1.4 Jacob's modified electivity index (D; Jacob's second selection index)

Jacobs (1974) derived a modification of ***E*** based on mortality rates for food items. He believed that the *D* is independent of food relative abundance. This index is calculated using formula:

$$D_i = \frac{r_i - p_i}{r_i + p_i - 2 \times r_i \times p_i}$$

where D_i is the Jacob's modified electivity index, r_i is the relative quantity (portion, percentage) of the food item ***i*** in the digestive tract content, and p_i is the relative quantity of the food item ***i*** in the environment.

That index takes values of 0 under random feeding and deviates symmetrically between – 1 (avoided food) and +1 (preferred food). It can give the full range of values for any particular value of food availability in contrast to ***E***. On the other hand, it is only slightly less independent to sampling errors for rare species than ***E*** and is also inappropriate for quantitative comparison of index values from different samples except unusual (or probably near unreal) situation with only two food items (Vanderploeg & Scavia 1979a, b; Lechowicz 1982). This index
also belongs to the group of indexes which do not estimate any biologically meaningful value (Manly et al. 2002).

*7.2.1.5 Vanderploeg & Scavia's first selection index (*W*)*

This index is derived from the forage ratio. It is normalised so that the sum of all partial selection ratios for food items in a sample equals one. The ***W*** was developed to avoid main weaknesses of the Forage ratio and Ivlev's electivity index and to get better estimates of electivity under various conditions of relative prey abundance. It also allows to investigate the preferences based on size, taste and other factors (Vanderploeg & Scavia 1979a) and is derived from raw data, mortality rates of prey, filtering rates, feeding rates and electivity indexes. ***W*** is defined between 0 and 1 and is calculated using equation:

$$W_i = \frac{F_i}{\sum_{i=1}^{n} F_i}$$

where W_i is the Vanderploeg & Scavia's first selection index for prey item ***i***, F_i is the proportion of the available category ***i*** items that are used (or the fixed property of the feeding animal, as originally inscribed by the authors), and ***n*** is the number of food items.

The above presented formula is only one of the variety of ways how to calculate ***W*** and this index offers broad range of possibilities how to get the final value from different kind of data, but is more suitable for advanced investigators (see Vanderploeg & Scavia 1979a, b for more information). Another view on this index (Lechowicz 1982) is, that it is the same index as the Chesson's α presented in Chapter 7.2.1.7.

This index can be regarded as feeder's perception of the value of food item in relation to both its abundance and the other items available. It measures an invariant degree of preference, it has biological meaning and can be interpreted as estimating the probability (or some multiple of the probability) that the next resource used will be of a specific type (Manly et al. 2002). Confer & Moore (1987) found this index as one of the most appropriate in situations when the number of food items in diet and the relative abundances of food resources vary among samples. On the other hand, the ***W*** is dependent on the number of food items and gives values from 0 to 1, which is an unusual range in the field of food selection where values from -1 to +1 are most often used.

7.2.1.6 Vanderploeg & Scavia's relativised selectivity (E; Vanderploeg & Scavia's second selectivity index)*

The range of values possible to be obtained in ***W*** were one of the reasons why Vanderploeg & Scavia (1979b) proposed new index called ***E****, an index analogous to Ivlev's ***E*** based on the selectivity coefficient ***W*** and the number of available food items. The equation to calculate it is:

$$E_i^* = \frac{W_i - n^{-1}}{W_i + n^{-1}}$$

where E^*_i is the Vanderploeg & Scavia's relativised selectivity, W_i is the Vanderploeg & Scavia's first selection index for prey item ***i***, and ***n*** is the number of food items.

Advantages of the ***E**** are, that it includes a measure of the feeder's perception of a food's value as a function of both its abundance and the abundance of other items of food in the environment. It also covers a measure of the deviation from random feedings in rank order which makes the comparison of electivities from diverse sites meaningful. These properties were reasons, why Lechowicz (1982) appreciate this index the single best and most useful (although not perfect). Also Confer & Moore (1987) found this index as appropriate in field studies with high variability of the number of food items in diet and the relative abundances of food resources, similarly to ***W***.

The values range theoretically from -1 to 1 with zero value for random feeding, which could be an advantage. However, in practice, the value of +1 can be attained only under unrealistic conditions with one food item in the gut which does not occur in the environment with infinite types of food similarly to the Ivlev's index (***E***). This index is also markedly nonlinear and asymmetrical, but these characteristics are inescapable if the index should be stabilised under changes in relative abundance of food items. The maximum achievable preference is an increasing function of the number of food items. The vulnerability to sampling error for rare and moderately common food grows with increasing number of food items and only samples with the same food item number are comparable and this index is not treatable with parametric statistical analyses (Lechowicz 1982). Tokeshi & Daud (2011) additionally demonstrated in an experiment, that it also does not necessarily reflect the deviation from random feeding.

7.2.1.7 Manly-Chesson's index (α; Chesson's selection index; Manly's standardised selection index B1 and Manly's standardised selection index B2)

This index is a derivation of the stochastic model of prey encounter and capture (Manly 1974; Chesson 1978, 1983; Lechowicz 1982). Several variants of this index are widespread used in feeding ecology studies. They are variants of the formula:

$$\alpha_i = \frac{\frac{r_i}{p_i}}{\sum_{i=1}^{n} \frac{r_i}{p_i}}$$

where α_i is the Manly-Chesson's index of selectivity for food item ***i***, r_i is the relative quantity of the food item ***i*** in the digestive tract content, and p_i is the relative quantity of the food item ***i*** in the environment.

Manly et al. (2002) distinguish these indexes[23]:

1. Chesson's index:

$$\alpha_i = \frac{\frac{r_i}{p_i}}{\sum_{i=1}^{n} \frac{r_i}{\pi_i}}$$

where α_i is the Chesson's index of selectivity for food item ***i***, r_i is the relative quantity of the food item ***i*** in the digestive tract content, p_i is the sample proportion of available units in category ***i***, and π_i is the proportion of the population of available units that are in category ***i***.

2. Manly's standardised selection index with used resource units replenished:

$$B_{i1} = \frac{\frac{u_i}{k_i}}{\sum_{i=1}^{n} \frac{u_i}{k_i}}$$

where B_{i1} is the Manly's standardised selection index for food item ***i***, u_i is the number of units in category ***i*** in a sample of used units, k_i is the number of available units in category ***i*** in a sample of available resource units.

This form of the index is used when the number of prey (or food item generally) eaten is very small relative to that prey item's total population (or available food supply) or when prey (food) are replaced, as in laboratory studies.

[23] Some symbols for variables in these equations were changed according to needs of this publication.

3. Manly's standardised selection index with used resource units not replenished

$$B_{2i} = \frac{log(1 - f_i)}{\sum_{i=1}^{n} log(1 - f_i)}$$

where B_{2i} is the Manly's standardised selection index, f_i is the proportion of the available category i items that are used.[24]

In this equation, base-10 logarithm ($\log_{10}$) is recommended by Lechowicz (1982), but according to Chipps & Garvey (2007), any base of logarithms can be used. This form of the index is used when the number of prey (food) eaten is large relative to that prey item's total population (food supply) in the environment or when, in experimental studies, prey (food) are not replaced after being eaten.

Values of this index (or these indexes) are normalised so that:

$$\sum_{i=1}^{k} \alpha_i = 1,0$$

where k is the number of food items in the sample and α_i is a value of the Manly-Chesson's index (or B_{i1}, or B_{2i} respectively).

The expected value varies between 0 (complete avoidance) and 1 (complete positive selection) and it is a function of a number of food items. It means that values below 1/k (number of food items in the sample) indicate avoidance and values above 1/k indicate preference. The value of 1/k 0.5 indicate random feeding nonselective towards the particular food item i.

This index is nonlinear and changes in the occurrence of food in the gut and in the environment do not have the same effect at all values. Similarly to ***W***, the advantage is, that this index allows meaningful comparison between samples because it is unaffected by the relative abundance of food items (Lechowicz 1982) and the selection estimated has biological meaningful value (Manly et al. 2002). When comparing with other indexes, it seems to be one of the best choices for quantifying food items selection (Chesson 1983; Chipps & Garvey 2007). It is recommended to use the Manly-Chesson index for variable prey populations when the number of prey eaten and the number of prey remaining are greater than 10 (Manly 1974; Chesson 1983; Krebs 1989; Chipps & Garvey 2007). The problem of interpretation related to this index can occur when very rare food items (prey) are presented in the diet. They have a major effect on

[24] Manly uses more variants of variables in equations than other authors dealing with the selection problematics. It reflects more complex view on the selection and potential factors affecting the accuracy of the final value of selection index. The variables used and defined in these Manly's equations may look a bit confusing on first look, thus we highly recommend to study Manly's publications on this problematics to use the equations correctly. Otherwise, it could be better to use the simpler variant (α).

values for all other food because they exceed the sum of the ratios. This effect could accurately reflect the selection, or can be an artefact of small sample size. It must be also remembered that a high value does not mean automatically that the particular food is numerically important in the diet (Confer & Moore 1987).

7.2.1.8 Strauss' linear selection index (L)

The inadequacies of Ivlev's electivity index and the Forage ratio lead to the development of the linear food selection index (Strauss 1979). The calculation of this index is very easy using formula:

$$L_i = r_i - p_i$$

where L_i is the Strauss' linear selection index of the food item ***i***, r_i is the relative quantity of the food item ***i*** in the digestive tract content, and p_i is the sample proportion of available units in category ***i***.

Properties of this index include the – 1 (avoidance) to + 1 (maximal positive selection) range, the expected value of the index for random feeding is always zero (similarly to the ***E***, extreme values appear only when prey item is rare but consumed almost exclusively, or is very abundant in the environment, but rarely consumed and is approximately normally distributed). Following its characteristics, is should be preferable in most situations to the ***FR***, ***E, D*** and ***log Q*** (Strauss 1979). Strauss'***L*** can be suitable for describing the impact of predation on the population of prey (Confer & Moore 1987). Strauss (1979) did not only mention the positives of this index. He recognised that this index is vulnerable to sampling error for rare items (in the environment or in the diet), but the effect is smaller than in ***E*** and ***E'*** indexes and the problem is that the error is growing with growing value of food utilisation (Strauss 1979). In addition, ***L*** also suffers from the essential faults of ***FR***, ***E, D*** and ***log Q*** and cannot be used for comparison of samples with differing abundances in the environment or diet (Lechowicz 1982). It reflects only selection for the particular circumstance observed and do not estimate any biologically meaningful value (Manly et al. 2002).

8 Graphical techniques of data presentation

The information obtained by the gut content analysis was presented in a form of tables or numbers alone for a long time. This way of presentation makes difficult to interpret two or more indexes (and/or values) simultaneously and clearly on the first look. Graphical techniques attempt to overcome this problem by combining two or more diet measures in two- or more-dimensional space (i.e., bivariate plots). This concept of the diets characteristics representation based on the advantage of visual information can be easier to interpret than tables or numbers alone (Costello 1990; Cortés 1997; Welker & Scarnecchia 2003). Even further, by examining relationships between different diet measures, graphical techniques can be used to evaluate and interpret also more complex aspects of the fish feeding ecology and feeding behaviour, such like predator feeding strategies (specialisation versus generalised), relative prey importance, diet variability, diet breadth (dietary niche width respectively), and can indicate potential diet (dietary niche) overlap[25]. Graphical techniques afford the opportunity of a rapid, visual evaluation and comparison of data prior to further statistical analysis (Costello 1990; Marshall & Elliot 1997). They were developed and used mainly in the predatory feeding fish. These methods can be also used in combination with others techniques (as example, to identify the food items that stand out in quantitative terms or investigate specialisation/generalisation tendencies, Bennemann et al. (2006) recommend the use of the dominance in combination to graphic methods.)

8.1 The graphical method of Costello

This method developed by Costello (1990) relates the prey abundance (%***N***, ***%V***, or ***%m***) to the frequency of occurrence (***%F***). It was developed to evaluate the feeding strategy and prey importance. In praxis, each point on the graph represents the percent occurrence and abundance for a prey taxon (Fig. 16). Prey points occurring in positions close to the corners can be considered as follows: (1) prey points positioned close to 100% frequency of occurrence and 100% abundance are the dominant prey taxa; (2) points positioned close to 100% occurrence and 1% abundance indicate the predator takes many different prey taxa in low abundance (generalised diet); (3) points close to 1% occurrence and 100% abundance indicate a specialisation on certain taxa by some predators; (4) points close to the 1% occurrence and 1% abundance are the least important and probably consumed only accidentally. Diagonals can thus be drawn into the plot. They represent prey importance and predator feeding strategy. If the points

[25] Dietary or niche breadth and overlap are very complex problematics which cannot be sufficiently explained and described in this publication. There are too many approaches and methods dealing with niche in general and also with a partial, dietary niche. Graphical methods do not give us exact values of niche breadth or niche overlap. They just very clearly indicate potential trends which can be later analysed and tested using variety of indexes and other analytical and statistical methods explained in specialised literature.

are located along and below the diagonal labelled “prey importance” originating at the origin (0% to 0% = left bottom corner), this suggests that the feeding was homogeneous amongst the investigated fish. Conversely, if the points are spread along and below the second diagonal labelled as “feeding strategy” originating in the 0% to 100% (left upper) corner, it suggests that feeding was more heterogeneous and different prey taxa may be important because of their great abundance in a few predators or low abundance but high frequency of occurrence in many predators (adapted from Costello 1990).

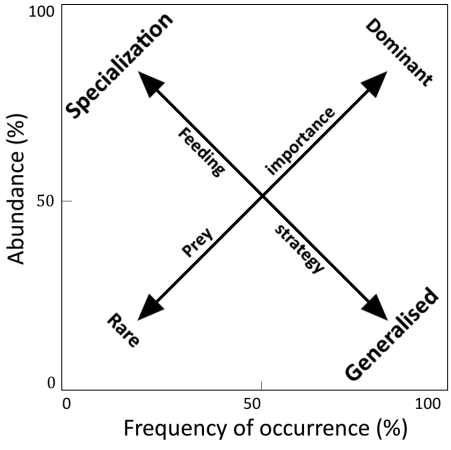

Figure 16 Explanatory diagram of the graphical method according to Costello (1990). (Redrawn and modified from Costello 1990 and Amundsen et al. 1996.)

This method is visibly subjective. The feeding behaviour is described by the position of the points within the plot and the interpretations of the position and dispersion depend on the investigator. In addition, more shortcomings of this method and difficult (or ambiguous) interpretations in some cases were pointed. As an example, the occurrence of a “generalised” diet, one with a wide niche breadth, as highlighted by a cluster of points within the bottom right corner, can be seen to be an unlikely occurrence. This was highlighted by Tokeshi (1991). Similarly, the occurrence of prey items of a high abundance but low occurrence (top left corner), is more likely to symbolize the occurrence of large organism as a rarity within the diet rather than a generalised diet for the species (Marshall & Elliot 1997), and data points indicative of generalised diet are not strictly confined to the lower right of the diagram, but may be distributed along the entire x-axis (Tokeshi 1991; Amundsen et al. 1996). Further, the sum of percent abundances of prey must be exactly 100. Hence it is not possible that several prey points will be clustered in the upper left of the diagram (Amundsen et al. 1996). These difficulties led to improvement and modification of Costello’s method and other variants of the graphical approach appeared. They also offer a different interpretation of the gut content data obtained and are based on the plotting of different diet characteristics. We describe them in the next chapters.

8.2 The graphical method of Tokeshi

Tokeshi (1991) developed a new method to eliminate the shortcomings of the Costello's method. His method uses the "mean individual feeding diversity" (D_i) plotted against the "population feeding diversity" (D_p) to indicate the feeding strategy of the species or different size classes. D_i and D_p, based on the Shannon-Wiener diversity index (H), were calculated according to the following formulas:

$$D_p = -\sum_{i=1}^{R} P_i \times \ln P_i$$

and

$$D_i = \frac{-\sum_{i=1}^{R} P_{ij} \times \ln P_{ij}}{N}$$

where D_p is the population feeding diversity, D_i is the mean individual feeding diversity, R is the total number of fish examined, P_i is the proportion of prey item i in the entire fish dataset ("population"), P_{ij} is the proportion of preyitem i in the fish j.

The data points for each species (or size group, bio-, or eco-type) are graphed and analysed (Fig. 17).

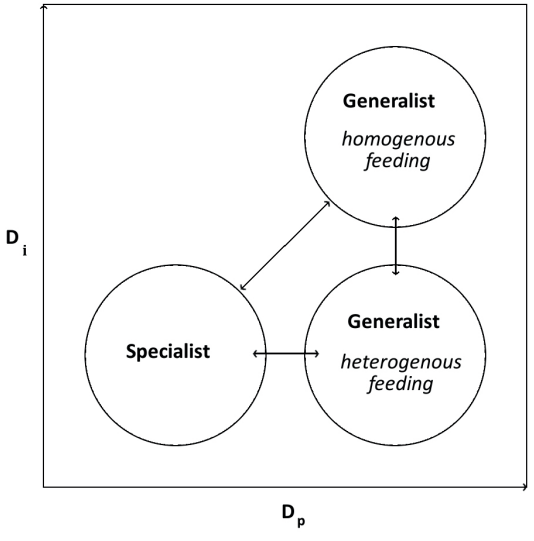

Figure 17 Explanatory diagram for interpretation of feeding strategy according to Tokeshi (1991). (Redrawn and modified from Tokeshi 1991.)

A population with low D_i and low D_p (the lower left corner in the plot) correspond to a specialist, whereas high D_i and high D_p (the upper right corner) correspond to a generalist with homogenous feeding regime. High D_i and low D_p (the lower right corner) indicate generalist with heterogeneous feeding regime and high D_i and low D_p is considered to be a rare occurrence (Tokeshi 1991).

This method seems to be more objective analysis than the Costello's method and gives a more objective analysis of the data (Marshall & Elliot 1997). However, this is the least used graphical method.

8.3 The graphical method of Amundsen

Another modification of the Costello's method conducted Amundsen et al. (1996) is very often used. To overcome the problems inherent in the Costello's method, the authors suggested to incorporate a new parameter, the "prey-specific abundance" (P_i), into the graphical representation of dietary composition (Fig. 18). P_i is defined as the percentage a prey taxon comprises of all prey items in only those predators in which the particular prey occurs, and is calculated as:

$$P_i = \left(\frac{\sum S_i}{\sum S_{ti}} \right) \times 100$$

where P_i is prey-specific abundance (expressed in numbers, mass, or volume) of prey i, S_i is the abundance of prey i in stomachs (expressed in numbers, mass, or volume), and S_{ti} is the total abundance of prey in predators that contain prey i (Amundsen et al. 1996).

The prey-specific abundance (P_i) is plotted against frequency of occurrence expressed in fraction rather than in percentage according original description of this method. Final plot is used to evaluate three important aspects of the fish diet: (1) prey importance (dominant versus rare), (2) feeding strategy (specialised versus general), and (3) niche width (see the Fig. 18 and the text below). Thus, this method enhances the ecological insight that may be derived from stomach contents data (Amundsen et al. 1996).

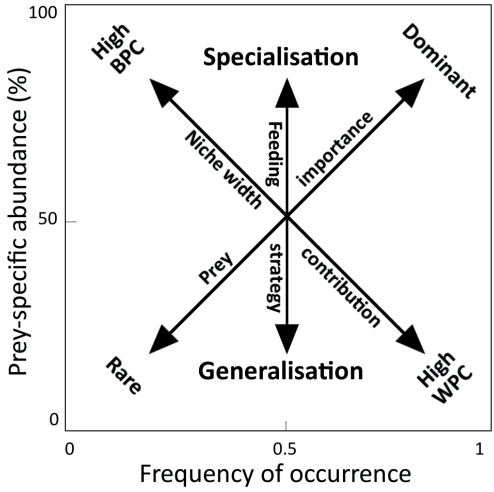

Figure 18 Explanatory diagram for interpretation of feeding strategy, niche width contribution and prey importance according to Amundsen et al. (1996). (BPC - between-phenotype component; WPC - within-phenotype component. Redrawn and modified from Amundsen et al. 1996.)

Information is obtained by examination of the point distributions. Important is the location of points along the diagonals and axes of the diagram as described in Amundsen et al. (1996) and in the Fig. 18:

1. the prey importance is concluded from the position at the diagonal from the left lower corner to the right upper corner. The dominant prey is positioned at the upper, and rare or unimportant prey at the lower end. It should, however, be emphasised that prey importance (or abundance) is not represented by a linear increase along the diagonal, but rather as a function of prey-specific abundance and frequency of occurrence;
2. the feeding strategy of the predator is evaluated according to the position related to the vertical axis. The predators have specialised on prey items positioned in the upper part of the graph, whereas prey positioned in the lower part have been eaten more occasionally (generalisation);
3. the niche width and the contribution of within-phenotype and between-phenotype component is estimated using the diagonal from the upper left to the lower right corner. Observations located to the upper right of the diagram (population specialisation) must necessarily be restricted to a single or a few points, reflecting a predator population with a narrow niche width. If there are no prey points in the upper right of the diagram, and all prey points are located along or below the diagonal from the upper left to the lower right, the predator population will have a broad niche width. In a population with a high between-phenotype component, different individuals specialise on different resource types and the points are positioned in the upper left corner, whereas in populations with a high within-phenotype component, most of the individuals utilise many

resource types simultaneously and the points are clustered in the lower right corner.

8.4 The graphical method of Cortés

Another popular and very often used graphical method is the Cortés (1997) modification of the Costelo's (1990) method. His new graphical method uses frequency of occurrence (***%F***), relative numerical abundance (***%N***), and mass (***%m***[26]) (or ***%V***) in a three-dimensional graphical representation of population-level stomach content data (Fig. 19).

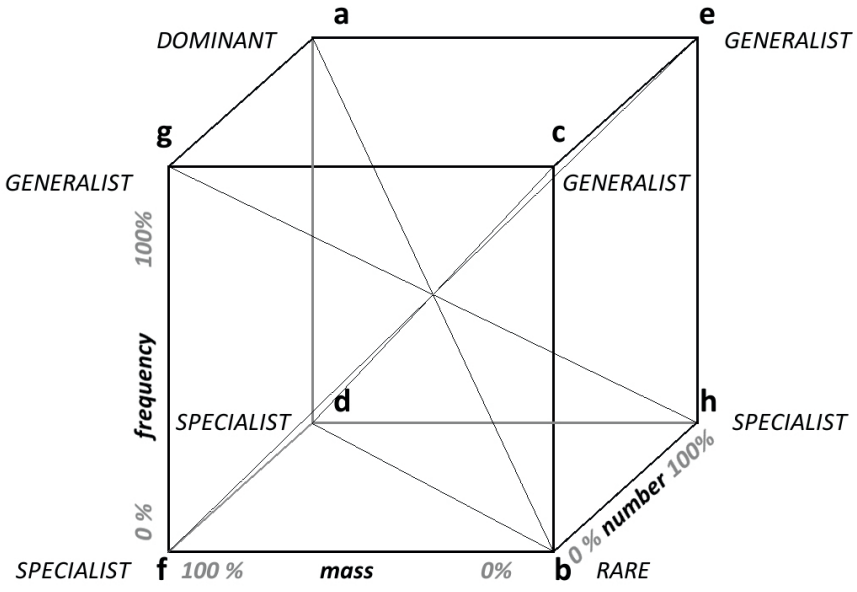

Figure 19 The three dimensional graphical representation of the fish gut content according to Cortés (1997). See text for definitions and explanation. (Redrawn and modified from Cortés 1997 and Gelwick & Matthews 2006.)

Interpretation is more difficult when comparing with the other graphical methods, considering the three dimensions and consequently much more possible point locations. According to Cortés (1997), each point on the graph represents the percent occurrence and abundance for a prey category. Prey points located close to 100% ***F***, 100% ***m***, and 100% ***N*** (point ***a***) are the dominant food taxon or category. Conversely, points located near the origin (point ***b***) of the three axes represent rare prey items. Any point located closer to the ***%N*** axis than to the ***%m*** axis along the horizontal plane indicates that counts contribute more than mass to the abundance of that item. Conversely, any point located

[26] ***%W*** as percentage of „weight" in original description

closer to the ***%m*** axis than to the ***%N*** axis along the horizontal plane indicates that mass contributes more than counts to the abundance of that item.

The other six vertices of the cube can be regarded as extreme cases pointing to either specialised or generalised diets. Thus, a cluster of points located close to 100% ***F*** and the origin of the other two axes (***%W*** and ***%N***; point ***c***)) would indicate a generalised diet (most predators take several different prey taxa in low abundance). In contrast, a point close to 1% ***F***, 100% ***m***, and 100% ***N*** (point ***d***) would indicate a specialised diet by a few predators, which would take large numbers of heavy items or items that make up a very large proportion of the total number and mass of stomach contents. A point located close to 100% ***N***, 100% ***F***, and 1% ***m*** (point ***e***) would be indicative of a light food item consumed by most predators. Conversely, a point close to 1% ***F***, 1% ***N***, and 100% ***m*** (point ***f*** in) would indicate a specialised diet by a few predators, which would take a few very heavy items or items that make up a very large proportion of the total mass of stomach contents. A point located near 100% ***F***, 100% ***m***, and 1% ***N*** (point ***g*** in) would indicate that most predators take a few heavy items or items that make up a very large proportion of the total mass of stomach contents. In contrast, a point close to 1% ***F***, 1% ***m***, and 100% ***N*** (point ***h***) would indicate a specialised diet by a few predators, which would take very large numbers of light items (Cortés 1997).

Diagonals can also be drawn on the three dimensional plot as proposed by Costello (1990) for his two-dimensional graphical analysis. They can visualize prey importance (dominant versus rare prey taxa) and predator feeding strategy (generalised versus specialised feeding). A line uniting points ***b*** and ***a*** would indicate increasing prey importance, and lines uniting points d and ***c***, ***f*** and ***e***, and ***h*** and ***g*** would all indicate a shift from a specialised to a more generalised feeding strategy (Cortés 1997).

Summary

1. Stomach content analysis is very important part of the feeding habit study, feeding ecology and, in general terms, a necessary step in research focused on more complex questions of freshwater fish ecology. Gut content analysis helps understand the problematics of fish species natural history, nutritional requirements, trophic, material and energy dynamics, food webs, food chains, material and energy transfers between and within ecosystems. It makes possible to explain interactions with other organisms such like predation or competition and contribute to the understanding of the ecological niche, ecosystem structure, community composition and population dynamics. We also cannot omit its value in practical fish conservation, evaluation and prediction of environmental changes and non-indigenous or invasive species impact, and in understanding food relationships in extensive or semi-intensive pond aquaculture.

2. This large diversity of potential use and exploitation of stomach content data is reflected in a variety of approaches, techniques and methods employed in the analysis of the fish digestive tract contents. These days, we know a very high number of gut content analysis methods. The history and evolution of these methods are quite long. Plenty of publications deal with the methods of gut content analysis of fish. These methods are also quite different regarding complexity and field of application. A number of publications and reviews analyse and evaluate advantages and disadvantages of these methods. Some authors actually try to find the best universal method, which is apparently a lost labour. In fact, it is sometimes difficult to get a relatively satisfying overview of the methodology of the gut content analysis alone without being engaged with studying literature for a long time.

3. We believe that simpler methods (e.g., frequency method, simple indexes) give us more robust results and better predictable potential error than methods that are more subjective and more vulnerable to various factors (e.g, bulk methods, point methods, compound indexes). Our opinion, even if based on results of reliable publications, may not be acceptable widely. Even though, we do not try to judge which methods are the best in this publication. Thus, we represent and describe majority of methods developed for purpose of fish gut content studying. As mentioned above, different methods have a different field of usage, different advantages and different limitations. They were often developed to avoid limitations of existing methods, or to obtain and/or interpret data different way and to help to answer a different question. Probably no one method is perfect and suffers from some weaknesses and constraints. Additionally, it is difficult to believe that it is possible to achieve ideal conditions, obtain ideal samples and provide perfect ecological research without any doubts and potential misinterpretations, especially in field research. Thus, researchers also need to know which methods is usable in a specific situation, or to answer specific question. It is also very important to know all the limitations of methods used in our research to avoid fundamental deficiencies which can waste the results.

4. Consequently, this publication is a try to present the problematics of freshwater fish gut content analysis digestedly. It can serve as practical handbook on individual procedures starting with sampling, through processing, identification, quantification, to final evaluating and interpretation of data obtained. We summarise and discuss pros and cons, possibilities, applications and limitations of different methods as they were uncovered and published to provide both - a theoretical background and a practical guide for a specialist in freshwater ecology, hydrobiology, fish biology, and ecology, employees in fishery and aquaculture.

Súhrn

1. Analýza obsahu tráviacich traktov je dôležitou súčasťou výskumu potravných návykov a potravnej ekológie, ale vo všeobecnosti tiež nevyhnutný krok vo výskume zameranom na komplexnejšie a zložitejšie otázky ekológie sladkovodných rýb. Analýza potravy pomáha lepšie porozumieť problematike biológie rýb, ich potravných a výživových nárokov, tok hmoty a energie, potravné reťazce a potravné siete, či prenos energie a hmoty v rámci ekosystémov a medzi ekosystémami. Umožňuje tiež vysvetľovať vzťahy medzi organizmami, napríklad kompetíciu, alebo predáciu, no pomáha nám chápať aj ekologickú niku, štruktúru ekosystémov, zloženie spoločenstiev a populačnú dynamiku. Nemôžme vynechať ani praktický význam výsledkov získaných touto metódou v ochrane rýb, vyhodnocovaní a predpovedaní vplyvov zmien v životnom prostredí na ryby a ďalšie organizmy s ktorými sú v interakcii, či dopady prítomnosti a šírenia nepôvodných a inváznych druhov a pre lepšie porozumenie potravným vzťahom v extenzívnej a polointenzívnej rybničnej akvakultúre.

2. Obrovský počet možností využitia údajov o zložení obsahu tráviacich traktov sa odzrkadľuje v neuveriteľne širokej palete prístupov, techník a metód, ktoré boli vyvinuté na ich štúdium. V súčasnosti poznáme veľké množstvo týchto metód, ktoré majú tiež bohatú históriu, postupne sa menili a vyvíjali. Opísané sú v množstve publikácií, mnoho ďalších ich hodnotí, porovnáva, analyzuje ich výhody a nevýhody. Niektorí autori sa dokonca pokúšajú nájsť najlepšiu, univerzálnu metódu (čo je však, samozrejme, márnou snahou). Vzhľadom na to je často ťažké získať relatívne dobrý prehľad o metodológii štúdia obsahu tráviacich traktov bez dlhodobého intenzívneho získavania a štúdia literatúry.

3. Veríme, že jednoduchšie metódy (napr. frekvencia výskytu, jednoduché indexy) nám poskytujú robustnejšie výsledky a sú zaťažené lepšie predpovedateľnou potenciálnou metodickou chybou, ako metódy, ktoré sú komplikovanejšie, viac subjektívne a citlivé na rôzne faktory (napr. metódy založené na odhade objemu, bodové metódy, zložené indexy). Náš názor, aj keď podložený relevantnými publikáciami, nemusí byť všeobecne akceptovaný. Okrem toho sa v tejto publikácii ani nepokúšame hodnotiť, ktoré metódy sú najlepšie. Predstavujeme a opisujeme tu väčšinu metód, ktoré boli vyvinuté na štúdium potravnej ekológie rýb pomocou analýzy obsahu tráviacich traktov. Ako uvádzame vyššie, rôzne metódy majú rôzne využitie, rôzne výhody a nevýhody. Často vznikli s cieľom vyhnúť sa chybám existujúcich metód, alebo získať a interpretovať údaje odlišným spôsobom, či zodpovedať úplne odlišné otázky. Pravdepodobne žiadna z metód nie je perfektná a musíme brať do úvahy jej slabiny a obmedzenia. Navyše je naivné veriť, že pri výskume tohto druhu (a najmä terénnom výskume) je možné dosiahnuť ideálne podmienky, získať ideálnu vzorku a zrealizovať Bezchybný, perfektný ekologický výskum bez akýchkoľvek pochybností a potenciálnych dezinterpretácií. Preto je nevyhnutné vedieť, ktorá metóda je použiteľná v konkrétnej situácii, resp. na zodpovedanie konkrétnej otázky. Potrebné je tiež poznať všetky slabiny metód, ktoré plánujeme použiť, aby sme sa vyhli základným hrubým chybám, ktoré by mohli znehodnotiť získané výsledky.

4. V tejto publikácii sa z vyššie uvedených dôvodov snažíme prezentovať problematiku štúdia tráviacich traktov sladkovodných rýb prístupným a zrozumiteľným spôsobom. Monografia môže slúžiť ako jednoduchý praktický sprievodca počnúc metódami beru materiálu, cez spracovanie vzoriek, určovanie a kvantifikáciu potravných komponentov, až po záverečné vyhodnotenie a interpretáciu získaných ýsledkov. Sumarizujeme a diskutujeme výhody anevýhody, možnosti, použitie a obmedzenia jednotlivých metód tak, ako boli odhalené a publikované. Publikácia preto môže poskytovať teoretický základ, ale aj praktickú príručku pre špecialistov v limnoekológii, hydrobiológii, ekológii a biológii rýb, či odborníkov v rybárstve a akvakultúre.

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