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COLLECTING AND PROCESSING NON-PLANKTONIC COPEPODS

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INTRODUCTION

Copepods (subclass Copepoda) are spectacularly abundant. There are probably more copepods on Earth than insects, with an estimated 1.37×10^{21} planktonic copepods inhabiting the pelagic realm, the largest biome on the planet (Boxshall and Halsey, 2004). As well as dominating the zooplanktonic communities in both marine and fresh waters, free-living copepods are also a major component of benthic communities. Copepods are usually the second most abundant metazoan taxon in the meiofauna of marine sediments, after nematodes, but they tend to dominate in coarse-grained sediments and on algae (Hicks and Coull, 1983). The maximum densities in intertidal sediments are usually in the order of 100,000-1,000,000 per m^2, but densities decrease with depth, so that in the deep ocean the number of benthic harpacticoids might be only 10,000 per m^2 (Coull et al., 1977). Copepods are also amazingly diverse in modes of life, and many have become parasitic or have a loosely symbiotic existence. They exploit a huge range of taxa as hosts, from marine algae to almost every aquatic phylum of metazoans, from sponges to vertebrates, including mammals. With the growth of aquaculture, many parasitic forms have emerged as commercially important pests, such as the sea-lice found on farmed finfish.

Copepoda currently contains about 14,000 described species placed in nine orders and about 210 family-level groups (Boxshall and Halsey, 2004). Calanoida is the dominant planktonic order and, while some are closely associated with the sea bed, almost all calanoids are planktonic and free-living. Cyclopoida (incorporating Poecilostomatoida) includes marine and freshwater planktonic and benthic forms plus a spectacular diversity of parasites and symbionts found on a wide variety of invertebrate and vertebrate hosts. Harpacticoida is the dominant group of benthic copepods, occurring in all types of sediments and in various biogenic substrates. Some harpacticoids have colonised the marine plankton, and a few live in symbiotic relationships. All members of order Siphonostomatoida are symbionts or parasites, and they are predominantly marine. The remaining five orders are considerably smaller: Misophrioida and Platycopioida consist of typically hyperbenthic species, many of which occur in anchialine habitats; Mormonilloida contains a few midwater oceanic species; and Gelyelloida inhabit continental groundwater communities. The Monstrilloida are parasitic as larvae and have free-living, non-feeding adults. Its validity has been questioned (Huys et al., 2007).

ECOLOGY

Planktonic copepods occur throughout the entire oceanic water column, from the epipelagic to the abyssal and hadal zones. They exhibit a variety of feeding behaviours from small-particle feeding and predation to scavenging on marine detritus. Different families tend to be concentrated in different depth zones, and some are found primarily in coastal rather than oceanic waters. Many epipelagic and mesopelagic species exhibit diurnal vertical migration, and this must be factored into any sampling regime. In freshwater lakes copepods of the families Cyclopidae and Diaptomidae (Northern Hemisphere, Africa, and northern South America) or Centropagidae (Australasia and southern South America) are dominant in the zooplankton.

Benthic copepods inhabit all kinds of sediments. Coarse-grained sandy sediments tend to be dominated by slightly flattened epibenthic species and have fewer interstitial and burrowing forms. In fine to medium sandy sediments, where the granulometry allows an interstitial existence, interstitial copepods can be abundant. Interstitial forms tend not to be present below a minimum critical grain size of approximately 200 μm. Shallow muddy substrates typically have both epibenthic and burrowing forms. Calcareous shell-gravel harbours yet another distinctive fauna.

Intertidal pools, which can be subject to extreme fluctuations in both temperature and salinity, are usually inhabited by the harpacticoid *Tigriopus* Norman, 1869. Copepods also live in phytal microhabitats; those inhabiting the fronds of macroalgae typically have a strong prehensile first swim-

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ming leg and can have a dorsoventrally flattened and bilaterally compressed body, or a long slender body. Increased complexity (surface area) of the algal substratum typically results in a concomitant increase in harpacticoid abundance and diversity. A range of benthic families can be found in sediment trapped in the holdfasts of macroalgae. Some specialized lineages are typically associated with mangrove-leaf litter where their entire life cycle is completed and appears to be synchronized with the decay of the leaves. The creeping nauplii and copepodids of some secondarily planktonic miraciids and thalestrids use floating macroalgal clumps or *Trichodesmium* colonies (Cyanobacteria) as a physical substrate in the open ocean.

In fresh water harpacticoids and cyclopoids can be found in every kind of habitat, from hot springs to glacial meltwater pools, and from the greatest depths of lakes to semi-terrestrial microhabitats. They also inhabit groundwater, cave pools of all sizes, and other subterranean habitats. Reid (2001) reviewed some of the more unusual habitats utilised by freshwater copepods including: ephemeral waterbodies, rock hollows, phytotelmata (pitcher plants, bromeliad pools, tree holes), leaf litter, damp moss, and moist soils.

Symbiotic and parasitic copepods live in association with the majority of marine metazoan phyla (Huys and Boxshall, 1991). Most are ectoparasitic, inhabiting the external body surface of their host as well as more sheltered microhabitats such as the oral-branchial cavity, nasal sinuses, and the eye orbit. Others have become endoparasitic, living in the gut of their worm or mollusc hosts, in the branchial chamber of tunicates, or in the internal canals of sponges. Copepods can be highly transformed, lacking any vestige of external segmentation, ornamented with elaborate arrays of processes or lacking appendages. The most characteristic feature that can be used to identify a parasite as a copepod is the presence of paired egg sacs in the females.

Collecting and Extraction Methods

Marine Benthic Copepods

Tube Coring.—Quantitative sampling of meiofaunal copepods in sediments is best carried out by tube coring. The standard corer used for copepods is a Perspex tube between 3.6 cm (surface area 10 cm^2) and 5 cm in diameter, but other sizes are used. The tube is usually about 30 cm long (Fig. 1A), and the lower edge that is driven into the sediment is externally bevelled to facilitate penetration. A suction device, such as a rubber stopper connected to a hose, helps to lessen friction when inserting the corer by reducing pressure above the core. A tight fitting stopper, or the plunger in a piston-corer, helps to retain the sediment when the corer is removed. In unconsolidated sediments a closing device for the bottom of the core is required, the simplest procedure being to dig out the tube and close it by hand. Corers can be marked to show sediment depth to facilitate subsampling of discrete depth horizons for quantitative study (Fig. 1E).

Known problems with corers include loss of animals related to the formation of a bow wave, and sample distortion due to compaction of the core (Higgins and Thiel, 1988). As the corer approaches the sediment surface the bow wave can cause flow around the corer, and epibenthic copepods can be caught in this flow and washed out of the sample. This problem is minimised by taking cores slowly and using flow-through designs. Compaction can occur as the corer is forced into the sediment. Friction between the wall of the corer and the sediment can compress the contained sediment and introduce bias into estimates of the volume of sediment sampled. Compaction is worse in certain kinds of sediment and can be minimised by increasing the diameter of the corer.

Subtidal samples in shallow water are best taken by divers who can position the samplers and take the core slowly; however, for sampling in deeper waters a variety of remote samplers has been developed, including box corers and grabs (Higgins and Thiel, 1988; Giere, 2009). Sediments with a well-developed flocculent layer or an easily re-suspended surface are difficult to sample but are least disturbed by 'deliberate' corers, which are typically lowered to the sediment surface by wire until the frame sits on the surface. The corer is released within the frame and slowly penetrates the sediment, with its action retarded by the action of a piston. This allows the retention of the flocculent layer and of water from immediately above the sediment. Such a system may carry a single corer (e.g., Craib, 1965) or multiple corers (e.g., Barnett et al., 1984).

Box Corers.—Box corers are used for sampling larger volumes of sediment. They are typically heavy devices that are designed to penetrate the sediment under their own weight. On reaching the sea floor the frame of the corer rests on the surface, and the box is released and slowly pushed into the sediment. As the corer is pulled up from the bottom a lever activates a spade that cuts through the sediment below the box, and an upper lid closes off the top of the corer. This effectively traps the sediment and the overlying water for retrieval to the surface. Flocculent materials are nevertheless inadequately sampled by box corers and there is evidence that box corers retain fewer meiofaunal organisms per unit area than 'deliberate' corers (Bett et al., 1994).

Grabs.—Grabs, such as the widely available Van Veen grab, are sometimes used for sampling meiofauna but cause significant disturbance to the sediment. They also tend to lose the overlying water during retrieval, with subsequent loss of fauna. Grab samples are not recommended for quantitative meiofauna studies (Higgins and Thiel, 1988).

Karaman-Chappuis Method.—This method was originally developed for sampling the hyporheic fauna in freshwater sediments (see below) but has proven very effective for collecting interstitial copepods from sandy beaches and other intertidal environments.

Light Traps.—Portable light-traps constructed of transparent Perspex and containing a chemoluminescent ampoule (Cyalume® Lightstick) as light-source are inexpensive to construct, easy to operate, and very efficient in attracting a wide variety of benthic and planktonic copepods (Holmes and O'Connor, 1988).

Extraction Methods.—Meiofaunal copepods are relatively easy to extract from sediments. A detailed account of the common extraction techniques was given by Pfannkuche and Thiel (1988). Optimal methods depend upon the median

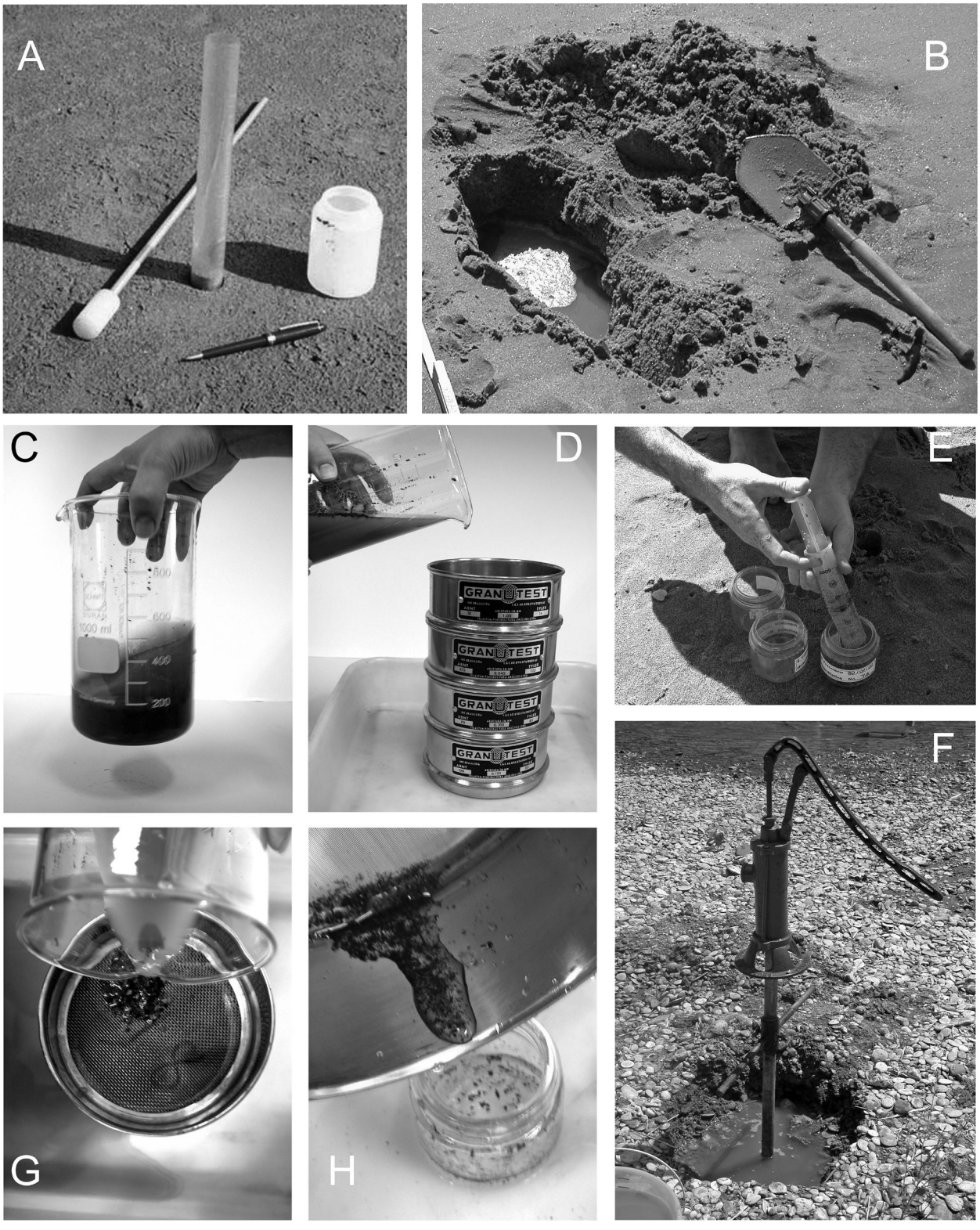

Fig. 1. A, Tube corer with simple piston plunger and sample container; B, Karaman-Chappuis method showing water flooding into hole; C, core sample being agitated in beaker with sea water; D, standard stack of sieves; E, graduated tube corer being subsampled into discrete depth horizons; F, Bou-Rouch pump; G, coarse sieve at top of stack removing larger pieces of detritus; H, target sample being washed from fine sieve into vial.

grain size of the sediment and on the state of the sample (i.e., whether it is preserved or fresh).

In medium to coarse sandy sediments, the sample is placed in a beaker or bucket with seawater and agitated (Fig. 1C), and then the supernatant is decanted into a stack of sieves (Fig. 1D), with the coarsest sieve at the top to remove larger pieces of detritus (Fig. 1G). The target size fraction retained on the finest sieve (45 or 63 μm) is then washed into the sample vial (Fig. 1H) and labelled. For quantitative studies the seawater decantation should be repeated twice and followed by freshwater extraction.

Decantation cannot be used for fine sand and muddy sediments; instead, copepods must be extracted by hand, an extremely laborious process, or by the use of flotation methods in which the meiofauna is first suspended by thorough mixing in a medium of a specific density, followed by short centrifugation to separate out the sediment. Isopycnic separation using silica sols is the fastest technique for extracting meiofaunal samples, saving up to 40% of the time needed to process a sample by sieving and decantation (see de Jonge and Bouwman, 1977). The medium most widely used is the colloidal silica polymer Ludox® (DuPont). Several types of silica sol are available, varying in price and chemical properties. The unmodified sols are very sensitive to divalent cations such as calcium and magnesium, and they rapidly precipitate to form a white gel on exposure to seawater. This can be prevented by using a freshwater pre-wash before the addition of the sol, or by using a more expensive modified sol. Aluminate stabilized sols are more resistant to gelling and can be used on brackish water samples without a pre-wash. Polyvinylpyrrolidone (PVP)-coated aluminate sols, such as Percoll® (Pharmacia), are required to work in full marine salinities but are expensive. Flotation methods are constantly being improved (see Burgess, 2001).

In samples with very high silt content, such as deep-sea samples, the addition of kaolin powder after fixation and centrifugation in a Levasil® solution (Bayer) can help to bind the finest, most easily suspended particles and yields a higher extraction efficiency (Heiner and Neuhaus, 2007). Samples with high plant-debris content, such as mangrove samples, may also require additional treatment. Such samples can be centrifuged for three periods of 10 min at 6000 rpm, mixed with kaolin to retain the finest sediment, and with magnesium sulphate ($MgSO_4$) of specific density 1.28 (Sunkur and Appadoo, 2011). In problematic samples, after first centrifuging with kaolin and Levasil®, a second centrifugation with magnesium sulphate is recommended, which increases extraction efficiency.

Sediments rich in clay tend to form clumps or concretions. Ultrasonic treatment has been tried to disperse concretions but is likely to cause excessive damage to the specimens. The addition of water softening agents such as Calgon® (Reckitt Benckiser), accompanied by prolonged stirring, has facilitated extraction of meiofauna, and Cedhagen (1989) recommended mixing the fixed sample with a solution of alkaline detergent Ajax® (Colgate-Palmolive). If the sample is then left for 20-40 min at 80-90°C, the detergent breaks down clay concretions and faecal pellets and facilitates subsequent sorting.

The extraction of minute copepods from samples and their transfer to vials or to slides or dishes for observation is best done with hooked needles or fine loops of thin wire known as Irwin loops. These consist of a wooden handle and a 3 cm length of non-corrosive, nickel-chromium wire ending in a tiny loop, and can be home-made (Schram and Davison, 2012). Pipettes can be used but there is a risk of the specimen getting stuck inside the pipette.

Phytal Copepods

Copepods living on marine macroalgae can be collected by placing a plastic bag over the fronds and detaching the holdfast of the alga from the substrate. In the laboratory such phytal copepods can be extracted by adding seawater to the plastic bag containing the alga and shaking it vigorously. This is followed by decantation through a fine-mesh sieve (45 or 63 μm). Extraction efficiency is enhanced by using an anaesthetic, such as isosmotic $MgCl_2$ (7% solution in full-strength seawater), or by using freshwater to administer a shock to the fauna. It is not advisable to fix macroalgae samples in formalin as this causes the copepods to cling more tightly to the fronds and reduce extraction efficiency (Huys and Boxshall, 1991). Some copepods show a more intimate association with their algal substrates, involving frond-mining and/or the production of galls. Various members of Thalestridae and Dactylopusiidae have nauplii and early copepodid stages that actively excavate the medullary tissues of brown and red algae. Rhodophyta contains the most heavily and routinely inhabited hosts.

Copepods can be abundant in mangroves. Fallen mangrove leaves at various stages of decay can be collected by hand and placed in plastic bags. In the laboratory they should be washed in filtered seawater and the washings passed through a 2 mm sieve to remove large particles before filtering through a 50 μm mesh net.

Freshwater Benthic Copepods

Karaman-Chappuis Method.—Both Karaman (1935) and Chappuis (1942) developed a method for sampling the hyporheic fauna in the water beneath gravel banks at the margins of rivers and streams. Now known as the Karaman-Chappuis method, it is the most basic technique for sampling copepods from interstitial water contained in fresh water sediments. The method involves digging a hole in the shore of a lake or on the sand or gravel bank of a stream. The hole should be dug away from the water's edge and has to be dug deep enough to reach the water level, which causes the hole to begin to fill with water (Fig. 1B). Interstitial water flows into the hole until it reaches equilibrium with the subsurface water table. The inflowing water can be scooped up in a plastic container and filtered through a fine mesh net. This qualitative method can be standardised to a limited extent by measuring the volume of water filtered or by continuing for a standard time interval.

Bou-Rouch Pump.—The Bou-Rouch pump is a hand piston pump (Bou and Rouch, 1967) modified from a design used originally for sampling in marine sediments. It is used to sample the shallow interstitial and is particularly useful for sampling streambeds that are difficult to penetrate using corers (Fig. 1F). It consists of an iron intake pipe, about

2-3 cm in diameter and 1.5-1.8 m in length. The hollow intake pipe has perforations in the shaft near the spiked tip and is driven 1-1.5 m into the sediment using a sledgehammer, taking care to protect the lip or thread at the top of the pipe from damage. Once at the desired depth the pump unit is fitted to the pipe and pumping can commence. A fine-mesh plankton net placed over the outflow of the pump will catch the copepods. In freshwater habitats this method is used for sampling phreatic as well as interstitial waters.

For deeper penetration into the interstitial the intake pipe can be extended to several metres, but the removal of extension pipes is often impossible. The hand piston pump can also be replaced by a tube running to an electric or diesel motorised pump.

Standpipe Corer.—Williams and Hynes (1974) designed a heavy-duty steel standpipe corer for sampling the interstitial fauna in the coarse substrates of stony streams. A long steel pipe with an internal diameter of 2.5 cm is constructed with a solid, pointed tip and two openings, each 10 cm long and shielded by welded wings near the tip. These openings can be opened or closed off by a core rod inserted within the standpipe once it has been driven to the required depth. The inner core rod is turned until the sample chamber is filled. The number of turns is determined by practice but is typically between 7 and 10. The sample chamber is then locked in the closed position to prevent loss of animals while the core is extracted.

Colonization Corer.—This method relies on the colonization of an artificial substrate placed in situ (Fraser et al., 1996). A standpipe is driven into the river or stream bed to the required depth, and a sediment-filled, acrylic colonization sleeve is inserted into the standpipe. Sediment is mixed according to specifications that mimic the already ascertained, vertical sediment particle distribution of the habitat. Following a colonization period of about 2 months, the acrylic sleeves are removed using a tripod and winch and wrapped in plastic film to prevent loss of fauna. The sleeves can then be cut into sections for analysis.

Frozen Sediment Corer.—The frozen corer method first paralyses the meiofauna in an electric field then freezes a core sample using liquid nitrogen (see Bretschko and Klemens, 1986). The freeze corer and two insulated copper rods are driven into the sediment using a post driver. An electric field (630 V, 60 Hz) is created between the two copper rods, using a portable generator, and is maintained for 10 min to paralyse the fauna. This prevents animals from evading the cold front as the liquid nitrogen is delivered. After the electric field is disconnected, the metal funnel is placed in the top of the standpipe, and 8-10 kg of liquid nitrogen is poured in, over a 10 minute period. The frozen sediment core is extracted using a tripod and winch and divided into sections, the volume of which is carefully determined. The need to transport a generator and liquid nitrogen supplies can limit the use of this apparatus.

Quantitative Sampling.—Fraser and Williams (1997) compared the four basic types of sampler used for quantitative studies of the hyporheic fauna: pumps, sediment cores, artificial substrates, and frozen sediment cores. They found that species richness did not vary significantly among the four methods, but that the colonization corer underestimated invertebrate density, and that there was a sampling bias in the pumping method.

Cave Copepods

Hand Collecting.—Care should be taken when sampling for any hypogean fauna not to over-collect and damage small, isolated populations. Collect only the minimum number of specimens required. Water in caves, including small pools, gour pools, and dripping or trickling water on calcite slopes, is best sampled by manual searching, simply looking for fauna and collecting specimens using a pipette. Crustaceans in thin or trickling films of water can be collected using small paint brushes, minimising damage. Care should be taken to avoid disturbing the sediment at the bottom of pools as this can quickly cloud the water. Pumps can also be used to draw water from pools for filtering; when this is done, the water should be returned to the pool. In larger pools a hand-held net fitted to an extendable handle is used to sweep through the water and stir the substrate on the bottom.

Traps.—Baited traps are used in the larger pools in anchialine and freshwater caves. They rely on the behaviour of the copepods and are particularly effective in sampling scavengers such as misophrioids. Traps can be constructed from plastic cylinders, with a fine mesh over one end (where the bait is also secured) and an opening at the other end. They should be weighted or secured to the substrate. These traps can be deployed and retrieved by divers or from the surface using lines and floats. A coarse plastic mesh (5 mm) covers the mouth of the trap to exclude larger animals. Pieces of hard garlic sausage are suitable for bait as they do not lose integrity over a 24-48 h deployment. On retrieval the contents of the trap are washed through a fine mesh net and fixed.

Copepods in Phytotelmata

The water in phytotelmata such as bromeliad leaf pools and tree holes can be sampled using siphons, pipettes, or turkey basters. Filter through a 50 μm mesh net and return the water to the hole or pool.

Copepods in Springs

Drift Netting.—Placing a drift net at the point of issue of a spring can be effective. A fine-mesh net is fixed in place and left to capture fauna as it is washed out of the ground. Drift nets can be used in combination with cave divers for sampling springs and accessible resurgences. As the divers enter the spring the drift nets are anchored in place; they are removed when the divers exit. As the divers advance up the flooded gallery, they agitate the silt on the bottom and walls of the gallery, dislodging fauna that is then washed out and into the nets.

Funnel Nets.—Water enters cave systems by percolation through cracks and fissures in the roof and walls. Such trickles can be small, but after heavy rainfall these can become quite active and they often contain epikarst fauna washed out of the surrounding rock strata. Trickle water can be directed through a funnel into a plastic container that has holes covered with fine net (mesh size 50 or 60 μm) cut in

two sides to retain copepods in the container while allowing excess water to flow out (Pipan, 2005). Such devices can be left to sample for extended periods of up to a month. Each trickle sample should be fixed and analysed separately.

Moss and leaf litter around springs and seeps, and from other damp environments, can be collected in sealable plastic bags. Copepods are extracted by adding water, shaking vigorously, and filtering the wash water through a 50 μm mesh net.

Symbiotic Copepods

Fish Hosts.—Copepods are parasitic on all kinds of fishes, from hagfishes and elasmobranchs to teleosts, and in all salinity regimes. Host information is vitally important in the study of parasitic copepods, so every effort must be made not to mix host species after capture because parasites may be transferred by accident while in the net. Fish used for parasitological examination should be killed and then frozen or fixed (if they cannot be examined) without delay. If it is necessary to transport the fish, they should be stored individually in plastic bags because of the possibility of ectoparasitic parasites being dislodged. Check in the laboratory for detached parasites in the bag. The fish should be examined externally for parasitic copepods in the following sequence (Kabata, 1985): examine the skin, fins, eyes, and nares (nostrils). Look for signs of external parasites, such as lesions, subcutaneous haemorrhages, and missing scales. Some copepods produce pouch-like invaginations by burrowing under scales along the side of the host, or into the walls of the alimentary canal, often in the rectal area. The nares should be opened and examined as they are a favoured microhabitat for parasites, such as species of Bomolochidae in marine fishes and of Ergasilidae in freshwater fishes. Remove the operculum from one side exposing the gill cavity, then excise the gill arch and examine the gill filaments for attached copepods. The gills are a favoured microhabitat for copepods, which may be concealed in the space between the hemibranchs. Finally, observe the walls of the gill cavity and inner surface of the operculum. Repeat this process on the other side, then open the mouth and examine the roof of the buccal cavity and around the tongue and teeth. Soaking the body in physiological saline solution for 30 min can dislodge small ectoparasitic copepods and the sediment from the soaking should be examined on a dissecting microscope.

Ectoparasitic copepods typically attach by means of clawed appendages. Care must be taken not to break off the claws when removing the parasite from its host, as these often provide useful taxonomic features. Chalimus larvae of siphonostomatoid fish parasites are attached by means of a frontal filament that is anchored to the surface of host; these larvae need to be picked off individually. Fish parasites are often coated with mucus from the host. The mucus can be removed using fine paintbrushes when the copepods are alive. After fixation, mucus can be drawn off by placing the parasites in a 16% glycerol solution and tumbling them for 6 h, after which they should be washed in distilled water before returning to the preservative.

Mesoparasitic copepods, for example members of Pennellidae or Sphyriidae, typically have large metamorphosed females that live with their heads embedded in the musculature of their hosts, forming branching, anchor-like structures. A few pennellid genera, such as *Peroderma* Heller, 1865, are almost entirely located within the host, with only a small opening to the exterior remaining through which protrude the paired egg sacs of the copepod. The presence of paired egg sacs, whether the eggs are arranged in a single series like a stack of discs, or more irregularly in multiple series, is an excellent indication of the presence of a copepod. The best way to extract a mesoparasite with its cephalic holdfast intact is to excise a large steak of the host, sufficiently large to enclose the full estimated extent of the holdfast, and place it in 50 ml of saturated potassium hydroxide (KOH). Cover it so that it cannot evaporate and leave for one or more days at room temperature, checking every day. The KOH digests the host tissues surrounding the holdfast so that it can be teased away using dissecting needles. This process also digests the internal tissues of the copepod but the empty exoskeleton is intact and can be used for taxonomic study. It is necessary to remove any tissue samples for molecular studies before beginning this procedure. Micro-CT methods offer a non-invasive way to visualise the embedded parts of mesoparasitic copepods (e.g., Schwabe et al., 2014).

A few copepods, members of Philichthyidae, are effectively internal parasites of fishes. They typically inhabit subcutaneous spaces associated with the sensory canals of the lateral line and skull bones of marine actinopterygian fishes. Their presence is indicated by swellings in some instances but in others there is no external sign of infection, so philichthyids are hard to find and are under-reported. They have traditionally been found by stripping the skin off the head and operculum of the fish, and examining the pore canals using a microscope, but a technique involving cutting the head into pieces, soaking and washing those pieces, then double netting the debris has been shown to be highly effective (Madinabeitia and Nagasawa, 2013). A genus of ergasilid copepods inhabits the urinary bladder of freshwater fish (Rosim et al., 2013).

Invertebrate Hosts.—Copepods utilise a huge range of invertebrate host taxa, and A. Humes developed an extraction technique that was to prove suitable for coaxing the copepods out from many host types. Humes and Dojiri (1982) found that maceration followed by rapid washing of freshly collected coral colonies or fragments usually yielded very few xarifiids. The same is true for the extraction of asterocherid and dinopontiid copepods from their sponge hosts. The Humes method, as applied to the extraction of xarifiid copepods from hard corals, is as follows.

> Immediately on collection in the field each colony or fragment of coral is isolated in a plastic bag. In the laboratory the coral and sea water are placed in a bucket to which sufficient 95% ethanol is added to make an approximately 5% solution. The coral is left in this solution at ambient temperatures for several hours or over night. Then the coral is thoroughly rinsed by shaking well and the wash water is poured through a fine net (120 holes per 2.5 cm, each hole approximately 100 μm square). The copepods are then picked from the sediment retained in the net.

It appears that the dilute alcohol, together with the accumulating products of decomposition, stimulates the copepods to leave the polyps of the coral host, and they fall to the bottom of the container. This method can be applied to many other host groups, such as soft corals, bivalves, echinoderms and sponges. Shortening the time of exposure

to ethanol to about 20-30 min results in a smaller yield but ensures that most copepods are still alive and therefore can be preserved adequately for DNA extraction. The key is to isolate the host individually in a plastic bag or metal bucket (in the case of sea urchins). Host information is so valuable that a return to the common nineteenth century host designation of "mixed invertebrate washings" would be highly undesirable.

Sea squirts, both solitary and compound, frequently serve as hosts for copepods, many of which are probably commensals inhabiting the pharynx or atrium of their hosts and sharing the food material brought in by the host's feeding activity. Some species are parasitic and can be confined to cysts within the tissues of the host in the branchial circulatory sinuses, for example, or in the subendostylar blood vessels. Copepods inside solitary sea squirts are often visible through the transparent tunic of the live host, a careful dissection should be made when dealing with preserved hosts. In colonial tunicates, copepods often occupy the spaces between zooids within the communal tunic.

Copepods parasitise most groups of molluscs, from chitons to cephalopods. For external parasites, such as those found on the gills of bivalves, washing the hosts in a dilute seawater-ethanol mix may dislodge the parasites into the wash water. Because copepods may also be found within the intestinal canal and even within the hepatopancreas, however, it is necessary to dissect the internal organs after examination of the outer surface, mantle cavity, gills, and ctenidia. Some copepods, such as members of Chitonophilidae, are highly transformed, consisting of a globular body and a branching rootlet system penetrating the host, and are difficult to recognise as copepods. The endoparasitic copepod *Nucellicola* Lamb, Boxshall, Mill and Grahame, 1996 was first discovered when gastropods (dog whelks) were placed in beakers of water searching for cercariae larvae of digenetic flukes, but nauplii were instead found in the water. They were released from an endoparasitic copepod that had such extremely reduced morphology that it no longer even had egg sacs (Lamb et al., 1998).

Polychaetes serve as hosts to 17 families of copepods, most of them parasitic. Some families are mesoparasites and, while the external ectosoma is conspicuous, the endosoma extending within the host may be large and encircle host organs. Careful dissection is required to remove such parasites intact. Scale worms (Polynoidae) are hosts of herpyllobiids, and it is necessary to search beneath the elytra (scales) for signs of parasitisation. Many copepods inhabit the burrows of burrowing polychaetes and echiuran worms, and some live within the secreted tube of tubicolous worms. Sampling burrows can be done effectively using a pump like a yabby-pump to extract the burrow water and then filtering it through a fine-mesh (50 μm) net.

Burrowing crustaceans, like callianassid shrimps in marine or crayfish in fresh waters, often host copepods within their burrows. Sampling burrow water collected by a pump system like the yabby-pump and then filtering it through a fine mesh net is effective at catching these copepods. Only one family of copepods, Nicothoidae, is fully parasitic on crustaceans, most inhabiting the brood pouch of the host. In peracaridan hosts this is the marsupium, and female nicothoids are typically globular in form and of a similar size to the host eggs; in ostracods they inhabit the brood chamber within the bivalved carapace of the host (Boxshall and Halsey, 2004). In live material the parasites are sometimes visible through the marsupium wall, but in fixed material it is best to remove the host's eggs to search for all life cycle stages of the parasite. Land crabs can host harpacticoid copepods within their branchial chambers (Humes, 1958).

Preservation

Material required for molecular analysis should be frozen or fixed in 95% or absolute ethanol. Such material should not be exposed at all to formalin or to IMS, which contains methanol. Other fixatives such as Lugol or DESS (Dimethylsulphoxide, EDTA and saturated NaCl solution) also allow for both morphological and molecular analysis on the same material.

To preserve meiofaunal copepods, a few drops of acid Lugol's solution can be added direct to the sediment sample up to a final concentration of 2-5% (Nicholas and Trueman, 2009). The fixed samples should be stored in a cool place away from the light. The medium should be exchanged for ethanol for long-term storage; re-fixing every 6 months is recommended. Extended immersion in this fixative, results in the material darkening over time.

DESS can be used to fix marine copepods and it seems to be more effective than the most traditional fixatives in preserving specimens for morphological and molecular analysis (Yoder et al., 2006). An advantage of this fixative is that samples can be maintained in this medium for indeterminate periods of time without re-fixation. Copepods can also be immersed directly in RNAlater® solution, an aqueous, non-toxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA. They can be stored indefinitely in this fixative at −20°C or below.

It is best to use one fluid for fixation and another for long-term preservation and storage (see Martin, 2016 for the differences between fixatives and preservatives). Most fixatives have a rapid effect on the tissues but can lead to excessive hardening when used as a preservative (Huys and Boxshall, 1991). Copepods are conveniently fixed in 5% buffered formalin solution. This concentration reduces the tendency of formalin to make copepods brittle. If higher concentrations are used then the addition of 2-5% propylene glycol helps maintain flexibility of the material. The formalin solution must be buffered at a minimum pH of 8.2. Suitable buffers are borax (sodium tetraborate) or hexamethylene tetramine, which are added at 200 g/l.

After fixation in formalin, copepods are commonly transferred to 70-80% ethanol or IMS (Industrial Methylated Spirit) for long-term storage even though this tends to lead to brittleness. The alcohol also destroys colour rapidly, so notes or photographs should be taken of any colour patterns. Ethanol produces a milky white precipitate when mixed with sea water and creates a deposit on the surface of specimens, so briefly rinsing material in distilled water prior to transfer is recommended.

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