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Ethanol concentration and sample preservation considering diverse storage parameters: a survey of invertebrate wet collections of the Natural History Museum Vienna

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Abstract

The beginnings of the invertebrate wet collections of the NHM Vienna date back more than 200 years. Since that time the jars have been controlled irregularly and replenished with 75% ethanol. A comprehensive analysis of the actual ethanol concentrations in the various invertebrate wet collections has not been performed so far, despite the fact that ethanol evaporation in such alcohol-water mixtures was to be expected. The resulting low concentrations might have damaged the material, especially the DNA. In the present study we tested altogether 634 glass jars of the five collections (Arachnoidea, Crustacea, Evertebrata Varia, Mollusca, Myriopoda) for ethanol concentration. Furthermore, a subsample (n = 197) was investigated concerning formaldehyde traces. Finally, the presence of DNA amplifiable by PCR was tested in 25 individuals from ten jars. The ethanol concentration was on average 65.7% and no significant differences according to jar sizes (30–100, 200–300, 500/1000 ml) or closure types (ground glass stoppers, plastic screw caps) were found. Yet, significant differences were present among various collections; especially low concentrations were found in the Evertebrata Varia jars (mean: 54.3%). The reasons for differences in ethanol concentration can in our opinion be explained by the presence/absence of climate control, the frequency of working with the collection, different focusses of maintenance strategies, the age of collections and – last but not least – differences among collections, which were regarded as negligible so far. Formaldehyde residues were found in 26% of the jars tested, with a maximum of 1200 mg/l. From the Evertebrata Varia collection individuals of *Ascaris* sp. (n = 15) and *Fasciola hepatica* (n = 10) were selected for the DNA tests. The oldest samples with PCR success were from the beginning of the 19th century, albeit some of them had currently low ethanol concentration or contained formaldehyde in the preservation fluid. From our PCR success we infer that a good conservation shortly after death is more important than the specific age of a sample.

Key words: ethanol, formaldehyde, scientific collection, storage conditions, DNA quality, curatorial recommendations

Zusammenfassung

Im Naturhistorischen Museum Wien wurden die Alkoholsammlungen der Evertebraten vor mehr als 200 Jahren begründet, seither wurden die Sammlungsgläser unregelmäßig kontrolliert und mit 75%igem Ethanol aufgefüllt. Aufgrund der besonders starken Verdunstung des Alkoholanteils in dieser 75%igen Mischung ist ein Absinken der Alkoholkonzentration im Lauf der Zeit zu befürchten, was zu Schädigung des Materials, insbesondere der DNA, führen kann. Eine systematische Untersuchung der Alkoholkonzentration in den verschiedenen Sammlungsteilen der Evertebratensammlung wurde bisher noch nicht durchgeführt.

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In der vorliegenden Arbeit wurden 634 Gläser der Sammlungen Arachnoidea, Crustacea, Evertebrata Varia, Mollusca und Myriopoda auf ihre Ethanolkonzentration getestet. Weiters wurde ein Teil der Gläser (n = 197) auf Spuren von Formaldehyd getestet. Das Vorhandensein von DNA, deren Qualität für PCR-Amplifikation ausreichend ist, wurde in 25 Individuen aus zehn Gläsern getestet. Der gemessene Alkoholgehalt betrug im Mittel 65,7 %. Es gab keine signifikanten Unterschiede zwischen Glasgrößen (30–100, 200–300, 500/1000 ml) und Verschlussarten (Schliffstopfen oder Plastikschraubdeckel). Allerdings zeigten sich signifikante Unterschiede zwischen den einzelnen Sammlungen, besonders niedrige Konzentrationen lieferten Gläser der Sammlung Evertebrata Varia (Mittelwert: 54,3 %). Die Unterschiede gründen sich unserer Meinung nach auf die Aufbewahrung bei unterschiedlichen Klimabedingungen in den Sammlungsräumen, der Nutzungsintensität, Pflegeschwerpunkten, dem Sammlungsalter und – nicht zuletzt – sammlungsbedingten Unterschieden, die bisher als vernachlässigbar galten. Aus der Evertebrata Varia Sammlung wurden Individuen von *Ascaris* sp. (n = 15) und *Fasciola hepatica* (n = 10) für die PCR-Tests ausgewählt. Die ältesten in den Tests positiven DNA-Proben stammen aus dem beginnenden 19. Jh. und enthielten teilweise niedrige Alkoholkonzentrationen oder auch Formalinrückstände. Diese Ergebnisse lassen vermuten, dass eine gute Konservierung kurz nach dem Tod für den PCR-Erfolg wichtiger ist als das konkrete Alter der Probe.

Introduction

Natural history collections are the basis and source of a wide range of scientific information (e.g. on systematics, phylogenetics, phylogeography, pest invasions, conservation, environmental contamination). The potential quantity of information that can be gathered from objects increases with the advances made in techniques and methods.

The paramount interest, therefore, must focus on an optimal preservation state of the objects. This became a light bulb moment to us recently, when we needed a dried specimen that was discarded in our collection two-hundred years ago, as it was classified to be worthless because it had lost all legs and appendages necessary for determination. Today we would be able to use it, due to the advances in techniques and e.g. molecular methods. Several kinds of investigations unknown at the time of collecting nowadays may help to clarify open questions. E.g., tissues from a monkey stored for more than 100 years in a museum revealed insights into the spread of the human immunodeficiency virus (HIV); tissues from birds stored for an equally long time increased our knowledge about the relationships between avian and human influenza viruses; and decades of egg collecting for museums later revealed the effect of DDT on eggshell-thickness (Suarez & Tsutsui 2004). Nonetheless, today's main purpose of scientific collections remains to address questions of systematics, taxonomy and evolutionary research.

The quality of information depends on the quality of preservation and maintenance of the collections (Kruckenhauser & Haring 2010). For long time, the only method of preservation was drying, until in 1662 William Croone for the first time preserved specimens in hermetically sealed glass-vials full of spirit (Simmons 2013). This technique truly became popular: even the deceased Admiral Lord Nelson was preserved in a barrel of brandy for later burial in England, after his death at the battle of Trafalgar in 1805 (Simmons 2013). Wet collections – as a second method of preservation alongside the dry specimens from earlier times – are proved to be used in our museum at the earliest in 1797, particularly with the establishment of a helminth collection (Schreibers et al. 1811; Fitzinger 1868; Sattmann et al. 1999; Stagl & Sattmann 2013). Their main advantage was to preserve the genuine shape of soft parts of organisms. For almost hundred years ethanol was the all but only preservative (occasionally, dried tissues were

stored in turpentine oil for its clearing effect – POLE 1813). Then formaldehyde was discovered to be a disinfectant (LOEW 1888; ARONSON 1892; TRILLAT 1892) and a fixative taking effect quicker than ethanol, with a better preservation of macroscopic structures and colour (BLUM 1893 a, b). On specific order of the former director Dr. Steindachner (1834–1919) formaldehyde was used in our museum as early as 1896 (letter to collector Sykora; STAGL 2002), although it was especially painful to the collector: "This formalin is a hellish substance, it burned on my […] grazed fingers like fire" (STAGL 2002). In the 20th century isopropanol enlarged the list of preservative fluids and replaced ethanol to some extent. During the last twenty years, against the background of increasing genetic research, a wide variety of chemicals and mixtures of chemicals was suggested or tested as preservatives that especially preserve the genetic information (NAGY 2010). The prevailing preservative fluid in most of the wet collections at present is still ethanol, to some extent formaldehyde. However, the preservative fluid is just one element influencing the quality of stored DNA (KRUCKENHAUSER & HARING 2010).

One disadvantage of wet collections is the risk of evaporation, particularly in ethanol, where evaporation first lowers the alcohol concentration to an extent destructive for the molecular integrity of the tissues, before the tissues fall dry. An adequate alcohol concentration is important for the quality of preservation, especially with regard to molecular genetic analyses (e.g. DNA sequencing). Formaldehyde, in contrast, alters the molecular structure of the tissues permanently without changing their shape. It has a much higher antiseptic strength than ethanol (e.g. MAZZOLA et al. 2009), which usually persists, although evaporation, polymerisation to the insoluble paraformaldehyde and oxidation to formic acid take place. But formaldehyde also fragments and modifies DNA chains, making them more or less inaccessible to molecular systematic analyses (WIEGAND et al. 1996; WILLERSLEV & COOPER 2005; KRUCKENHAUSER & HARING 2010). Special PCR protocols – including a pre-treatment repair step – only can reduce the damage caused by formaldehyde (SKAGE & SCHANDER 2007). It is the second disadvantage of wet collections, especially today when molecular research forms a major contribution to science.

In the Invertebrate Collection (excluding insects) of the Natural History Museum in Vienna – NHMW (3. Zoologische Abteilung, Naturhistorisches Museum Wien; http://www.nhm-wien.ac.at/forschung/3_zoologie_wirbellose_ohne_insekten), we hold several ten thousand vials with objects preserved and maintained in ethanol with a proposed concentration of 75%. A considerable number of these objects date back to the early 19th century. Since the 1950ies all vials are usually controlled in a bi- or tri-annual cycle, but only for visible deficiencies of liquid. Usually this is done intuitively. Until now, the alcohol concentration in the individual jars was not measured. New jars, however, were always filled with 75% ethanol, old jars occasionally refilled or – if fluid was missing – replenished with 75% ethanol. With regard to the disproportionally high alcohol evaporation in ethanol-water mixtures, we expected that the concentration in most of the old glasses had dropped over time. We further wanted to assess whether the jar size influenced this decrease, or whether the plastic screw caps introduced in the collections ten years ago performed differently than the glass stoppers. From a small amount of jars it is known that they contain specimens that had been transferred from formaldehyde to ethanol. Yet, many more jars probably still contained unknown amounts of formaldehyde, as information on the use of formaldehyde as a fixative was mostly considered not to

be noteworthy. In the present study we performed a comprehensive analysis of our wet collection. We tested (1) the ethanol concentration in a sufficient number of samples, (2) the influence of glass size and stopper type on alcohol concentration, (3) the influence of climatized conditions in the new storages, (4) for traces of formaldehyde in a part of those samples. Furthermore, we performed (5) a first evaluation of DNA quality by means of PCR tests in a selected subsample and discuss it's results considering age and alcohol concentration. We discuss the results considering possible initial fixation conditions and maintenance procedures as well as influence of handling. Finally, we provide recommendations for advanced collection maintenance.

Material & Methods

Samples for ethanol and formaldehyde analysis

For the physical and chemical analysis, different jars of all five collections constituting the 3rd Zoological Department (invertebrates exclusive of insects) were selected (Table 1). Jars were selected from each collection to form subgroups of 20 jars with the same jar size and closure type (this was not possible for Evertebrata Varia, because not enough screw cap jars were present in this collection), and we wanted each subgroup to largely represent the respective collection. To that end jars were selected from the most prominent parts of every collection. For Crustacea / Decapoda these prominent parts are the old collection (collected at the end of the 19th century and the beginning of the 20th century), material collected during the 1970ies (mainly by G. Pretzmann), and material collected 1985–2005 (mainly by P.C. Dworschak). Myriopoda were chosen from the taxa Diplopoda and Chilopoda. The Arachnida collection is represented by jars mostly from Araneae and Scorpiones, with a small number of Opiliones and Pseudoscorpiones, the material was collected between 1885–2000. From the Mollusca collection jars from all shelves were chosen to meet the basic requirements of the sampling strategy (jar sizes, closure types). The height of fluid level was not considered as a parameter since, in general, such differences were minor (several millimeters at maximum). Moreover, in those rare cases when one of the randomly selected jars showed a considerable fluid loss, it was handed over to maintenance and a jar next to it was chosen. The frequency

Table 1: 614 jars statistically analysed for ethanol concentration, some of them (number in brackets) were additionally tested for formaldehyde. Old jars are ground glass stopper jars, new jars are screw cap jars. Only the latter have information on their cubic capacity imprinted on the glass.

Collection	Small jars 30–100ml		Medium jars 200–300 ml		Large jars 500/1000 ml	
	old	new	old	new	old	new
Evertebrata varia	65(29)	1(1)	--	28(-)	40(-)	--
Mollusca	20(17)	20(13)	20(9)	20(6)	20(9)	20(7)
Myriopoda	20(-)	20(-)	20(-)	20(-)	20(-)	20(-)
Crustacea	21(9)	21(9)	20(8)	20(8)	20(9)	18(9)
Arachnoidea	20(8)	20(6)	20(8)	20(7)	20(8)	20(7)

of use also was no selection parameter for the jars, because we miss a major prerequisite for tracking all jars that have been handled: we do not have a complete inventory. All collections have the same maintenance timetable, but exceptions may occur. The collection Evertebrata Varia comprises invertebrate taxa not included in the other four collections, and neither insects. Altogether 634 jars were included. The ethanol concentrations of 10 small *Ascaris* jars used for DNA analysis were excluded from the statistical analyses because small Evertebrata Varia jars were already overrepresented.

Jars of the old type are made of soda-lime glass, have ground glass stoppers and may date back to the 19th century. We infer the latter from the jar and stopper style ("hand-made" appearance, e.g. irregular forms of the lip and/or the stopper handle), combined with certain styles of attached labels and inscriptions. This type of jar was purchased until the 1990s from various suppliers. Their cubic capacity is not imprinted on the glass, therefore Table 1 contains only approximate information on old jar sizes. Jars with screw caps have been used since 2003 for storing all newly acquired samples and to replace defective old jars (Fig. 4). During tests from 2000 to 2002, they showed tight sealing (almost no evaporation when containing 75 % ethanol), no corrosion from the alcohol, undamaged caps and were easier to open and as well easier to close tightly than old jars. They are wide mouth bottles made of soda-lime glass with knuckle thread according to DIN 168 (DEUTSCHES INSTITUT FÜR NORMUNG 1998). The screw cap is made of black polypropylene (PP) with a thin inlay for tight closure made of LDPE (low density polyethylene). The dealer VWR International (pers. comm.) kindly provided detailed specifications (Table 2) and information on the material. Both plastics are manufactured by the Lyondellbasell Industries AF S.C.A. group, the PP is Moplen® HP400R (density 905 kg/m³, yield stress 32 MPa, softening at 154 °C), the LDPE is Lupolen® 1800 H (density 919 kg/m³, yield stress 9 MPa, softening at 88 °C).

The collections' storage conditions

Wet collections of Arachnoidea, Crustacea, Evertebrata Varia and Myriopoda are mostly stored in various non-climatized working rooms (Figs. 1–3) of the 3rd Zoological Department on the second floor of the building. Comparable rooms in the building showed a temperature of 20–26 °C, except for July/August (25–30 °C), and a humidity of 20–50 % (record from May 2004 to April 2005). The Mollusca wet collection has been moved into the new underground storage (approximately 200 m², and 600 m³ cubic

Table 2: Specifications of screw cap jars used in our department, according to the information of the dealer VWR International.

Cubic capacity of the jar (ml)	height (mm)	diameter (mm)	mouth (mm)	thread DIN 168	screw cap thickness (mm)	inlay thickness (mm)
30	68	36	24	GL 32	1.8	1.2
50	75	44	24	GL 32	1.8	1.2
100	92	50	32	GL 40	1.9	1.2
250	113	70	45	GL 55	1.9	1.0
500	154	84	45	GL 55	1.9	1.0
1000	180	103	57	GL 68	1.8	1.2

Figs 1-3. Storage conditions in the wet collections under investigation: 1, Arachnidoidea, 2, Evertebrata Varia, and 3, Mollusca.

Fig. 4. Jars in the Evertebrata Varia collection with their labels, and a modern screw cap jar (50 ml). The two jars to the left probably date back to the 19th century, because of their "hand-made" appearance. The small oval labels are numbers allocated in the time of Bremser, the small rectangular label at the bottom of the second jar from the left (NHMW 4485) was allocated in the time of Diesing. NHMW 4477 lost these labels, the mark (glue) of the oval one is visible on the stopper.

capacity) in 1990–92 where it has remained since then (Fig. 3). The climate conditions are standardized to 16 °C an a relative humidity of 50 %, allowing for a variation of ± 1 °C and ± 10 %. The ventilation number is 0.33, i.e. a third of the air volume (and any ethanol vapour in it) is replaced every hour.

Ethanol and formaldehyde analysis

Analysis of ethanol concentration was conducted with a portable "Density Meter" (Anton Paar GmbH, Graz, Austria). The liquid is taken up inside its density sensor (a U-tube made of borosilicate glass) with a lifting piston. The resonant frequency of this system reveals the liquid's density. A total volume of 3 ml liquid from the sample is taken; approximately 0.5 ml thereof remain in the intake tube. After every measurement the instrument was rinsed once with 75% ethanol to cleanse the instrument of dirty ethanol and because initial tests showed that small amounts of liquid remained in the density sensor after the measurement. That remaining liquid altered the next measurement unpredictably, depending on the ethanol concentration of the last measurement. In a test series, 10 jars containing 30% to 77% ethanol were measured twice, in the first run without rinsing the instrument. The results biased between -0.2% and +0.6%. Accordingly, what initially appeared to be 63.5% was in fact 64.1% in the second run, but 64.5% was 64.6%. Pure water, for instance, decreased an ethanol reading of 77.7% to 77.0%.

187 of the 614 jars tested for ethanol concentration (plus 10 *Ascaris sp.* jars) were also tested for formaldehyde (Table 1). Formaldehyde concentration was analysed with Merckoquant ® test strips ranging 10 – 20 – 40 – 60 – 100 mg/l (100 mg/l corresponding 0.012% formaldehyde concentration). This test also reacts to other aldehydes, but with a lower sensitivity and a different coloration. The test was performed according to the manufacturers´ instructions, including comparison of the colour of each test strip with the colour scale after 60 s. The difference in colouration of the 0 mg/l and 10 mg/l values was small (and the test strip continued to change colour after the required time), therefore not all of our 10 mg/l results may be accurate. The samples of *Fasciola* and *Ascaris* used for the DNA analysis were included and are part of the formaldehyde results of Evertebrata Varia. For some of the jars in the test, we expected positive results because we knew the collectors used formaldehyde for fixation and that this was later substituted by alcohol.

Samples for DNA analysis

To test the condition of DNA by PCR (polymerase chain reaction), altogether 25 ethanol-preserved specimens of the following taxa were analysed: 13 *Ascaris lumbricoides* Linné, 1758 from 9 glasses (NHMW 16752, 6955, 6943, 6947, 6942, 6946, 6952, 6944, 6958), 2 *Ascaris suum* Goeze, 1782 from one glass (NHMW 17279), and 10 *Fasciola hepatica* Linné, 1758 from 10 glasses (NHMW 4491, 4487, 4475, 19905, 4486, 10146, 4501, 4499, 4492, 4477; Fig. 4). The samples were selected from two genera represented frequently in our collection with a high variability concerning the time of acquisition and for which a variety of primers have been published.

Method of DNA analysis

DNA was extracted according to the manufacturer's instructions using the Qiagen DNeasy All Tissue Kit (Qiagen) with an incubation time of up to 24 h depending on visible progress of tissue digestion. The final elution volume was 30 µl. Optimal amounts of template DNA were determined empirically (1–10 µl of the DNA solution in 25µl PCR reaction volume). Control extractions without tissue were carried out to detect potential contaminations. PCR was performed with primers taken from the literature that amplify fragments of various gene sections in sizes between 89 bp and 350 bp (Table

3). The primers Asc1 / Asc2 and Asc10F / Asc11Rmod were designed for Secernentea (Nematoda), whereas Alum96F / Alum 183R are specific for *Ascaris lumbricoides*, TremFmod / TremRmod for Trematoda, and FASC_f / FASC_r for *Fasciola hepatica*. PCR was performed on a Mastercycler gradient thermocycler (Eppendorf) in 25 µl containing 2.5 µl of PCR buffer, 3 mM of Mg^{2+}, 0.2 mM of each nucleotide, 0.5 U of Q5 High-Fidelity DNA Polymerase (Biolabs) and 0.5 µM of each primer. PCR profiles comprised an initial heating step at 94 °C for 3 min followed by 45 cycles: 30 s at 94 °C, 30 s at annealing temperature and 60 s at 72 °C. Negative PCR reactions were repeated three times with different concentrations of template. After three unsuccessful PCRs they were recorded as negative. After the last cycle, a final extension of 7 min at 72 °C was performed. Control PCR reactions to detect potential contaminations were carried out with: (i) control DNA extractions, and (ii) with distilled water instead of the template. The authenticity of fragments was confirmed by sequencing some of the PCR products (some fragments were of very low concentration). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc.) and sequenced directly in both directions (using the amplification primers) at LGC Genomics (Berlin, Germany). GenBank accession numbers of sequences: KF798182–KF798190. The short ITS1 sequence can be retrieved from the authors on request.

Statistical analysis of ethanol concentration

Data from the Evertebrata Varia collection were analysed separately because their sample lacked small and large jars of the new type as well as medium jars of the old type (Table 1). Additionally, summary statistics already showed a clear difference between the Evertebrata Varia collection and the other collections.

Mollusca, Myriopoda, Crustacea and Arachnoidea collections were analysed together, their ethanol concentration being influenced by the following three factors: jar size, jar closure type and collection. The factor collection comprises several parameters, e.g. climate conditions, maintenance protocol, specimens inside (quality of fixation) or use

Table 3: Primers used for DNA analysis. Mitochondrial (mt) and nuclear (nc) marker genes: cyt b = gene for cytochrome b (mt), 18S = 18S rRNA gene (nc), ITS = internal transcribed spacer (nc) rep = genomic repeat sequence (nc). bp = base pairs. Fragment sizes are given for each of the 5 primer pairs. * = modified after LOREILLE et al. (2001). ** = modified after HAIDER et al. (2012).

Primer	Sequence	Fragment size (bp)	Gene	Reference
Asc1	5'-GTTAGGTTACCGTCTAGTAAGG-3'	142	cyt b (mt)	LOREILLE et al. 2001
Asc2	5'-CACTCAAAAAGGCCAAAGCACC-3'	142	cyt b (mt)	LOREILLE et al. 2001
Alum96F	5'-GTAATAGCAGTCGGCGGTTTCTT-3'	89	ITS1 (nc)	BASUNI et al. 2001
Alum183R	5'-GCCCAACATGCCACCTATTC-3'	89	ITS1 (nc)	BASUNI et al. 2001
Asc10F	5'-CCATGCATGTCTAAGTTCAA-3'	147	18S (nc)	LOREILLE et al. 2001
Asc11Rmod	5'-CAGAAAATCGGAGCTTTGGT-3'	147	18S (nc)	this study*
TremFmod	5'-GGTTCCTTAGATCGTACATAC-3'	428	18S (nc)	this study**
TremRmod	5'-GTACTCATTCGAATTACGGAGC-3'	428	18S (nc)	this study**
FASC_f	5'-ATTCACCCATTTCTGTTAGTCC-3'	124	rep (nc)	KAPLAN et al. 1995
FASC_r	5'-ACTAGGCTTAAACGGCGTCC-3'	124	rep (nc)	KAPLAN et al. 1995

by researchers. We selected our samples to form subgroups of 20 with identical jar size, jar closure type and collection. Possible statistical outliers were identified and their status checked by calculating the mean of the subgroup excluding the outlier, then an upper/lower threshold adding/subtracting four times the corresponding standard deviation. Everything outside the thresholds was regarded as an evident outlier. Excluding them resulted in approximate Gaussian distribution according to Kuiper's test for all subgroups, which were submitted to all statistical tests without these outliers. Typically these subgroups would be analysed with a three-way ANOVA, but due to missing homoscedasticity between the subgroups, we had to settle for a non-parametric test. Since there is no special non-parametric equivalent to a three-way ANOVA, we chose to fix each factor in turn, analysing the remaining two factors with a Scheirer-Ray-Hare-test ($p<0.05$). As a post-hoc test for significant results, we used Nemenyi-tests ($p<0,05$). In the case of significant interactions of the two factors in the Scheirer-Ray-Hare-test, a graphical analysis was performed to identify the nature of the interaction, and the result of the Scheirer-Ray-Hare-test was adapted accordingly. Additional statistics were calculated in cases of significant results in the Scheirer-Ray-Hare-tests in order to establish whether all subgroups involved show an equal magnitude of differences. Thus, for ground glass stopper jars showing significant differences among collections, we calculated a Kruskal-Wallis-test ($p<0.05$) for small and large jars and an ANOVA ($p<0.05$) for medium jars. For screw cap jars showing significant differences among collections, we calculated a Kruskal-Wallis-test ($p<0.05$) for small jars and ANOVAs ($p<0.05$) for medium and large jars. For small jars showing significant differences among collections, we calculated one-tailed t-tests ($p<0.025$) for Mollusca and Crustacea jars and one-tailed Welch's tests for Arachnoidea and Myriopoda ($p<0.025$, no homoscedasticity).

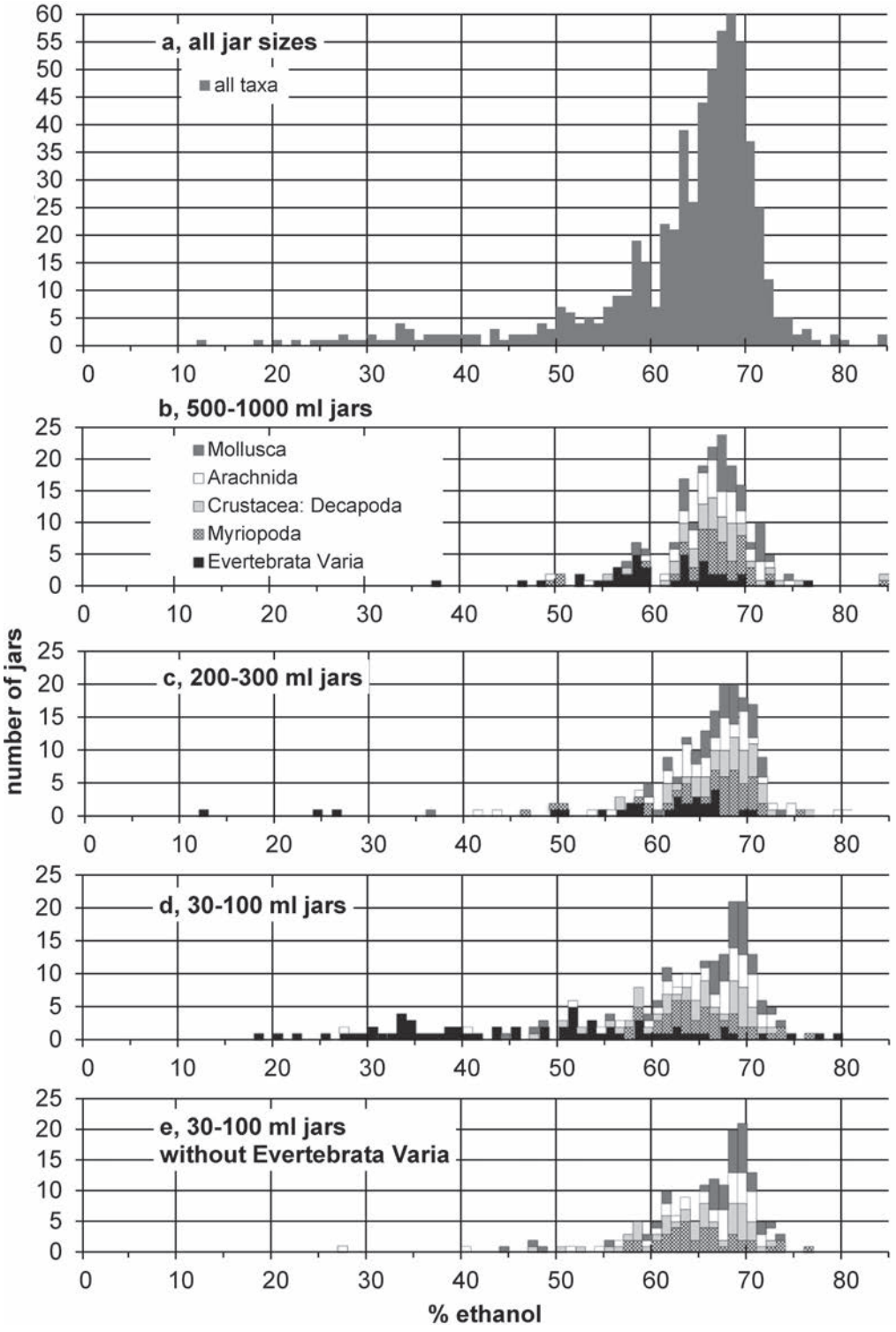
For small, medium and large screw cap jars of the collections of Mollusca, Arachnoidea and Myriopoda, respectively, we calculated ANOVAs ($p<0.05$) to analyse the increase/decrease of ethanol concentrations.

Results

General description of ethanol concentrations

Our main results is a predominantly good preservation in our wet collections (Fig. 5). 77% of all jars contained ethanol concentrations between 60.0 and 79.9%. Four collections had more jars in this range, i.e. Mollusca 90%, Myriopoda and Crustacea 88%, Arachnoidea 87%. The Evertebrata Varia collection, however, had only 38% of all jars within this range; especially small jars were below 60% ethanol concentration. The latter is described in detail below.

Fig. 5. Number of jars with a particular ethanol concentration (volume%, separate diagrams for: a, all jar sizes together; b, large (500/1000 ml); c, medium (200–300 ml); d, small jars (30–100 ml); e, small jars without Evertebrata Varia. ▶


a, all jar sizes
all taxa
b, 500-1000 ml jars
Mollusca
Arachnida
Crustacea: Decapoda
Myriopoda
Evertebrata Varia
c, 200-300 ml jars
d, 30-100 ml jars
e, 30-100 ml jars
without Evertebrata Varia
number of jars
% ethanol

Statistical outliers

Extremely low ethanol concentrations in the sense of statistical outliers were present in 2.4 % of all jars, which were from the following collections (Fig. 6): Myriopoda (6 outliers), Arachnoidea (5) and Mollusca (4). The Arachnoidea collection was the only one having such concentrations in the new screw cap jars. Extremely high ethanol concentrations were present in one sample of the Crustacea, Arachnoidea and Myriopoda collections, respectively. All outliers were excluded for the following statistical tests.

Ethanol concentrations and jar sizes

Nearly identical ethanol concentrations are evident in the three histograms for large and medium jars of all collections (Fig. 5b, c) and for small jars except from Evertebrata Varia (Fig. 5e). The summary statistics of these groups confirm this result: identical means (65 %, SDs 6–8 %) and medians (67 %). The Evertebrata Varia collection differs clearly from the other collections, and this applies especially, but not solely, to the small jars (Fig. 5d, e).

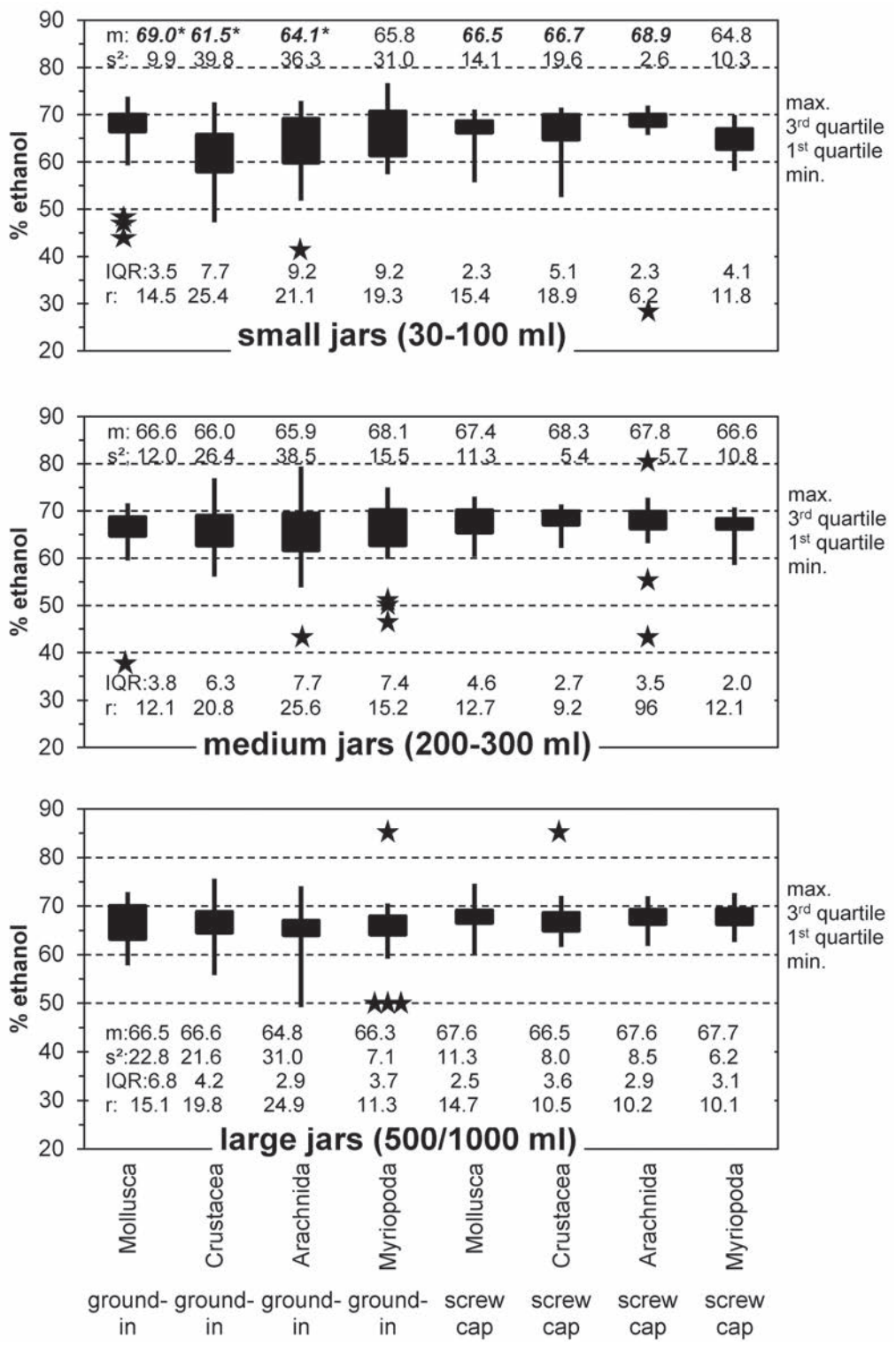
Within the other four collections there was no significant difference between small, medium and large jars ($p<0.05$; Table 4). The test, however, which also included the factor 'type of collection' as a statistical need, showed significant differences of ethanol concentrations among the four collections ($p<0.05$), forming a different pattern for each jar closure type (Table 4). All ground glass stopper jars of Mollusca and Myriopoda had significantly higher ethanol concentrations than those of Arachnoidea and Crustacea. All screw cap jars had significant differences: Arachnoidea > Mollusca > Myriopoda. These patterns are differently pronounced according to jar size: The values were significant only for small jars (both closure types, $p<0.05$; cf. Fig. 6). For medium jars (both closure types), differences were observed, but were statistically insignificant ($p<0,05$; cf. Fig. 6).

For the screw cap jars a significant interaction between the factors 'jar size' and 'collection' was present ($p<0,05$) (see plot in Fig. 7, right part). The Crustacea screw cap jars therefore had to be excluded from the above mentioned consideration for significant differences. For the remaining screw cap jars the increase in ethanol concentration with jar size of the Myriopoda jars (Fig. 7, right part) was significant ($p<0.05$), which was not the case for that in Mollusca jars. Moreover, the decrease in Aranchnida jars was also not significant ($p<0.05$).

Ethanol concentration and jar closure type

The new screw cap jars contained ethanol concentrations that were insignificantly different from jars with ground glass stoppers ($p<0,05$; Table 5). The latter were predominantly used for decades or even a century and were, needless to say, regularly refilled with 75 % ethanol; some received a complete exchange of ethanol. Yet they contained similar

Fig. 6. Ethanol concentration in jars with ground-in stoppers and screw caps, respectively, from four collections (no Evertebrata varia). n = 20, except for Crustacea: Decapoda (small: n = 21; large screw cap: n = 18). Boxes represent 10 samples. The upper and lower line represents 5 samples each, except where asterisks indicate extreme values (here they differ from the main bulk by more than 7 % ethanol concentration). IQR = interquartile range (v/v % EtOH), m = arithmetic mean (v/v % EtOH), r = range (v/v % EtOH), s^2 = variance; m, r and s^2 calculated without extreme values. Evertebrata Varia are not included because the sample sizes differ greatly, see Table 1. ▶


% ethanol
m: 69.0* 61.5* 64.1* 65.8 66.5 66.7 68.9 64.8
s²: 9.9 39.8 36.3 31.0 14.1 19.6 2.6 10.3
IQR:3.5 7.7 9.2 9.2 2.3 5.1 2.3 4.1
r: 14.5 25.4 21.1 19.3 15.4 18.9 6.2 11.8
small jars (30-100 ml)
max.
3rd quartile
1st quartile
min.
m: 66.6 66.0 65.9 68.1 67.4 68.3 67.8 66.6
s²: 12.0 26.4 38.5 15.5 11.3 5.4 5.7 10.8
IQR:3.8 6.3 7.7 7.4 4.6 2.7 3.5 2.0
r: 12.1 20.8 25.6 15.2 12.7 9.2 96 12.1
medium jars (200-300 ml)
m:66.5 66.6 64.8 66.3 67.6 66.5 67.6 67.7
s²:22.8 21.6 31.0 7.1 11.3 8.0 8.5 6.2
IQR:6.8 4.2 2.9 3.7 2.5 3.6 2.9 3.1
r: 15.1 19.8 24.9 11.3 14.7 10.5 10.2 10.1
large jars (500/1000 ml)
Mollusca
Crustacea
Arachnida
Myriopoda
ground-in
screw cap

concentrations as screw cap jars, which were all fitted with 75 % ethanol after 2003 (after their introduction in our collection).

The same test, which also included the influencing factor 'type of collection' as a statistical need, again revealed significant differences between collections for the small jars only, plus a significant interaction ($p<0.05$, Table 5).

Here, a general comparison was impossible because of the statistically significant interaction of the factors 'collection' and 'closure type'. A graphical analysis of the interaction (Fig. 7, left side) revealed two groups with respect to jar closure type: Both Arachnoidea and Crustacea had significantly ($p<0.025$) higher mean ethanol concentrations in screw cap jars, whereas Mollusca and Myriopoda had higher concentrations in ground glass stopper jars (significant for Mollusca, $p<0,025$). Within the two groups, differences of mean ethanol concentrations were also significant ($p<0.05$), i.e. Arachnoidea > Crustacea, and Mollusca > Myriopoda.

Table 4: Mean ethanol concentrations (volume %) within two groups of jars with different closure types: simultaneous comparison of jar sizes and collections. *Asterisks indicate significance $p<0.05$. Horizontal lines separate groups which show statistically significant differences. The result in parentheses must not be considered due to significant interaction between the factors jar size and collection.

factor levels	ground glass stopper	screw cap
30–100 ml	64.9	66.6
200–300 ml	66.6	67.8
5000/1000 ml	66.0	67.4
Mollusca (small+medium+large jars)	67.3*	67.17*
Myriopoda (small+medium+large jars)	66.7*	66.36*
Crustacea (small+medium+large jars)	64.6*	(67.19*)
Arachnoidea (small+medium+large jars)	64.9*	68.11*

Table 5: Mean ethanol concentrations (volume %) of three jar size classes: simultaneous comparison of closure types and collections. Level of significance (Scheirer-Ray-Hare-test) is indicated by asterisks: * $p<0.05$. Horizontal lines separate groups which show statistically significant differences.

factor levels	Small 30–100 ml	Medium 200–300 ml	Large 500/1000 ml
Ground glass (Moll+Myr+Crust+Arach)	65.1	66.6	66.0
Screw cap (Moll+Myr+Crust+Arach)	67.0	67.5	67.4
Mollusca (ground glass+screw cap)	67.6*	67.0	67.0
Myriopoda (ground glass+screw cap)	65.3*	67.3	67.1
Crustacea (ground glass+screw cap)	(64.1*)	67.2	66.5
Arachnoidea (ground glass+screw cap)	(66.5*)	66.8	66.2

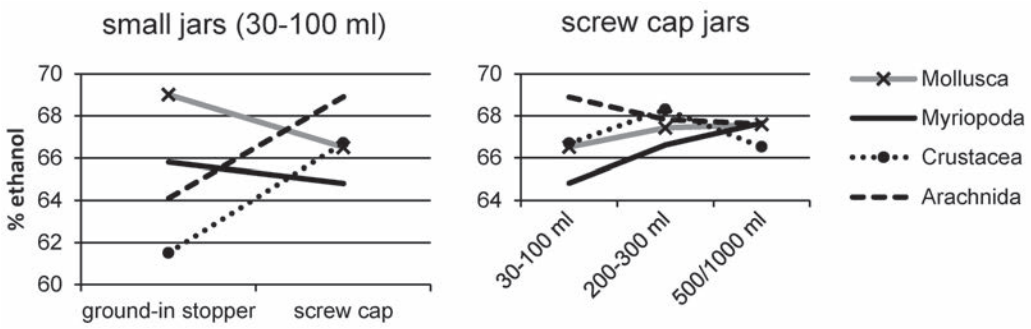


Fig. 7. Statistically significant interactions between the two influencing factors collection on the one hand and jar size (right) and closure type (left) respectively on the other hand. Crossing lines represent disordinal interactions, nonparallel lines ordinal interactions, parallel lines no interaction. Comparison of mean ethanol concentration (v/v%) by collection for (left side) all small jars and (right side) all screw cap jars. The section "screw cap" in the left part is identical to the section "30–100 ml" in the right part.

The ethanol variation was smaller in screw cap jars of each collection (Fig. 6). There were three exceptions: medium and small jars from the Mollusca collection and large jars from the Arachnoidea collection. In general, the screw cap jars had low variances and the ground glass stoppers jars high variances. Again, there were exceptions: among screw cap jars, small Crustacea jars showed a high variance and small Arachnoidea jars showed an exceptionally low variance. Within the group of ground glass stopper jars, the large Myriopoda jars had a very low variance (Fig. 6).

Ethanol concentration and collections

Statistical analysis of the four collections revealed a significant difference for either the factor 'closure types' or 'jar sizes' in all collections except Mollusca. For the Myriopoda, significant differences between jar sizes were detected, i.e. between small jars and the larger jars (Table 6, Scheirer-Ray-Hare-test, $p<0.05$), but no general difference between jar closure types. The Crustacea and Arachnoidea collections had highly significant differences between jar closure types ($p<0.01$).

Evertebrata Varia collection

Comparing the Evertebrata Varia collection with the combined data of the other collections yielded highly significant differences (Fig. 8). Additionally, the difference between small jars and other jar sizes was highly significant. The difference between the Evertebrata Varia collection and the combined data from the other collections was present within each of the three jar size classes (Fig. 8).

Ethanol colour

The colour of the fluid gave no clue as to the ethanol concentration. We found jars with completely colourless fluid containing 76% and 50% ethanol, respectively, and heavily coloured ones (yellow-brownish) containing 84% and 50% ethanol, respectively.

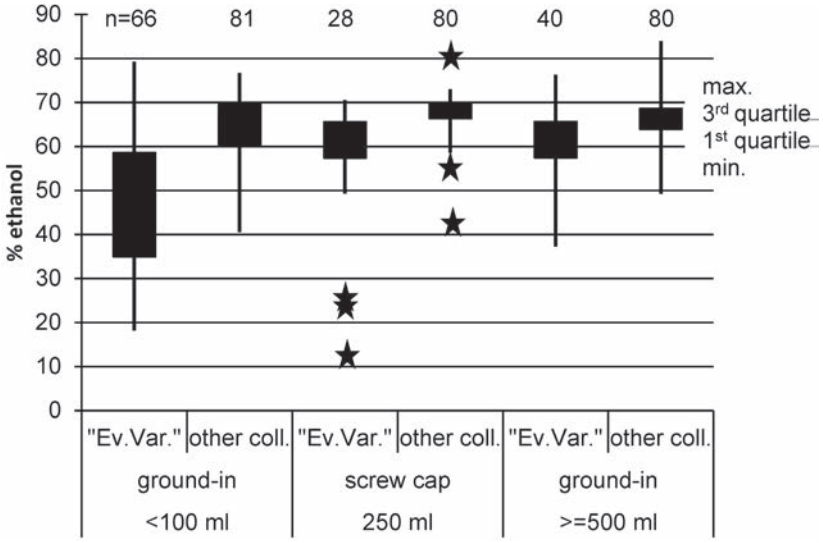


Fig. 8. Comparison of ethanol concentrations (v/v%) in the Evertebrata Varia collection and combined results of the other collections (Arachnida, Crustacea, Mollusca, Myriopoda). The upper and lower vertical lines represent lower and upper 25% of samples, except where asterisks indicate extreme values (25% minus extreme values).

Formaldehyde

All collections held jars containing formaldehyde (Fig. 9). Formaldehyde was more common in our wet collection than we expected. 26% of the jars tested for formaldehyde (n = 197) contained this fixative, while according to curatorial background information we expected 11% of the jars to be in this category. Thus, more than twice as many jars contained formaldehyde as expected.

The formaldehyde concentration in the jars, however, was low compared to the commonly used concentration of a 4% v/v formaldehyde solution (8200 mg/l). 56% of positive samples reached 10 mg/l, the detection threshhold of the test (in Fig. 9 indicated as 'detection limit'). The level of 20 mg/l (0.0024%), which is unambiguously visible

Table 6: Mean ethanol concentrations (volume%) within each collection: simultaneous comparison of different jars sizes and closure types. Level of significance (Scheirer-Ray-Hare-test) is indicated by asterisks: * $p<0.05$, ** $p<0.01$. Horizontal lines separate groups which show statistically significant differences.

factor levels	Mollusca	Myriopoda	Crustacea	Arachnoidea
30–100 ml	67.6%	65.3%*	64.1%	66.5%
200–300 ml	67.0%	67.3%*	67.2%	66.8%
500/1000 ml	67.0%	67.1%*	66.5%	66.2%
Ground glass stopper	67.4%	66.7%	64.6%**	64.9%**
Screw cap	67.3%	66.4%	67.6%**	68.1%**

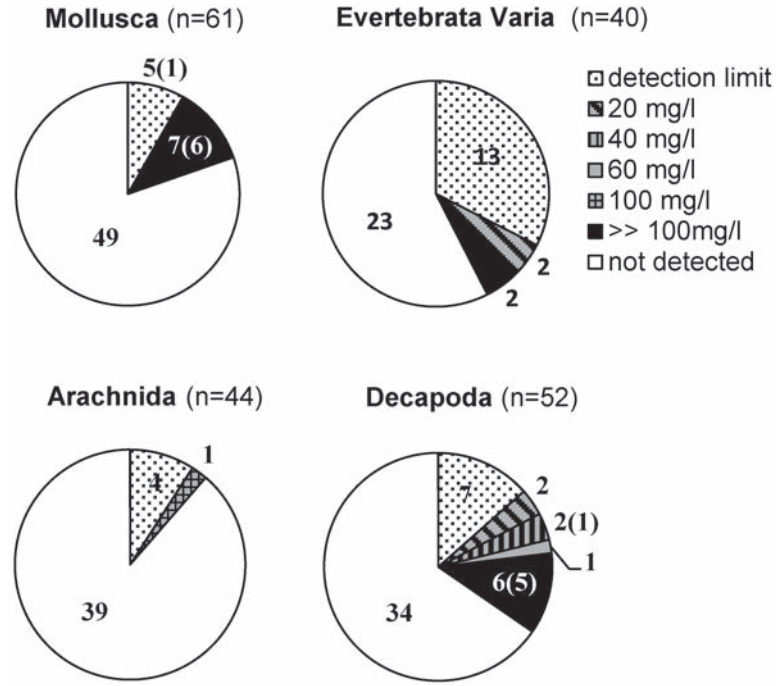


Fig. 9. Numbers of jars tested for formaldehyde (four collections), splitted for every collection according to detected formaldehyde concentrations. Numbers in parentheses indicate jar(s) in which we expected to find formaldehyde; they are given as a proportion of the preceding total number of jars.

on the colour scale, was found in 8 % of positive jars, and 30 % of all positive results exceeded the measurement range of 100 mg/l. Such samples were diluted and analysed again for samples used in the DNA analysis, the maximum formaldehyde concentration was 1200 mg/l.

DNA analysis

The results of the PCR success are summarized in Table 7. Five out of 15 *Ascaris* samples yielded PCR products of 147 bp (four of which were confirmed by sequence analysis). In those samples all three primer pairs used for *Ascaris* were successful. Furthermore, from nine *Ascaris* samples the short ITS1 fragment (89 bp) could be sequenced. Among 10 *Fasciola* samples, six yielded PCR products with the primer pair FASC_f / FASC_r (124 bp), while with TremFmod / TremRmod (428 bp) only three were successful (for details see Table 7). The ethanol concentration of positive samples ranged from 29.3 % to 77.5 %. Thus, even one of the samples with the lowest ethanol concentration yielded a PCR product, yet only the smallest fragment (89 bp). The oldest positive samples (both of *Ascaris* and *Fasciola*) were from the early 19th century. With four exceptions, no formaldehyde was detected in the preservation fluid of PCR positive samples: In three of them the concentration was only 10 mg/l, whereas in one the exceptionally high concentration of 400 mg/l was measured.

Table 7: Results of DNA tests using specimens of *Ascaris lumbricoides*, *A. suum* and *Fasciola hepatica*. Inv.No. = inventory number of the glass (Evertebrata Varia collection), time of acquisition for samples from the 19th century was taken from the inventory lists or reconstructed using information about labels (e.g., Fig. 4), biographic data of the collectors, specific publications or nomenclatorial characteristics of the labelling. Form = formaldehyde. + = clear PCR product of expected size. Seq = DNA sequence determined. Fragment sizes (in base pairs, bp) of the five primer combinations are provided in parentheses. For details of primers and abbreviations of genes see Table 2.

	Sample data			Preservation data			Primer combinations				
Taxon	Inv. No.	Sample code	Acquisition	Jar size ml	EtOH %	Form. mg/l	Asc1 / Asc2 cytb (142 bp)	Alum96 F / Alum183R ITS (89 bp)	Asc10F / Asc11Rmod 18S (147 bp)	TremFmod / TremRmod 18S (428 bp)	FASC_f / FASC_r rep (124 bp)
Ascaris lumbricoides	16752	Asc1	1996	100	55.0	0	Seq	Seq	Seq		
Ascaris lumbricoides	6955	Asc2	early 20th Cent.	50	64.1	0	+	Seq	Seq		
Ascaris lumbricoides	6943	Asc3	19th Cent.	50	72.9	0		Seq			
Ascaris lumbricoides	6947	Asc4	early 19th Cent.	50	51.9	0		Seq			
Ascaris lumbricoides	6942	Asc5	1901	100	76.3	0	+	Seq	+		
Ascaris lumbricoides	6942	Asc6	1901	100	76.3	0	+	Seq	Seq		
Ascaris lumbricoides	6946	Asc7	19th Cent.	50	29.3	0		Seq			
Ascaris lumbricoides	6946	Asc8	19th Cent.	50	29.3	0					
Ascaris lumbricoides	6952	Asc9	1826	50	67.8	0					
Ascaris lumbricoides	6952	Asc10	1826	50	67.8	0					
Ascaris lumbricoides	6944	Asc11	1826	50	69.9	0					
Ascaris lumbricoides	6944	Asc12	1826	50	69.9	0		Seq			
Ascaris lumbricoides	6958	Asc13	1927	50	64.6	0	Seq	Seq	Seq		
Ascaris suum	17279	Asc14	early 19th Cent.	100	46.7	0					
Ascaris suum	17279	Asc15	early 19th Cent.	100	46.7	0					
Fasciola hepatica	4491	Fasc1	early 19th Cent.	50	48.7	0					+
Fasciola hepatica	4487	Fasc2	1825	30	33.5	10					
Fasciola hepatica	4475	Fasc3	1931	30	62.3	400				+	+
Fasciola hepatica	19905	Fasc4	2004	50	77.5	10				Seq	+
Fasciola hepatica	4486	Fasc5	early 19th Cent.	30	34.0	10				Seq	+
Fasciola hepatica	10146	Fasc6	1957	100	62.8	1200					
Fasciola hepatica	4501	Fasc7	1866	70	51.1	10					+
Fasciola hepatica	4499	Fasc9	1859	30	67.2	0					Seq
Fasciola hepatica	4492	Fasc10	1852	50	45.3	0					
Fasciola hepatica	4477	Fasc11	early 19th Cent.	50	51.3	0					

Discussion

The results of our ethanol measurements indicate a generally good status of preservation in four out of five collections under investigation (exception: the Evertebrata Varia collection): the ethanol concentration means were approximately only 10 % lower than our target of 75 % and they are similar to those in other museums. At the Museum für Naturkunde Berlin, means of 64-69 % were measured (NEUHAUS et al. 2012), at the University of Kansas Natural History Museum the value was 59.3 % (their target is 70 %, WALLER & SIMMONS 2003), 63 % at the Oxford University Museum of Natural History (target 70 %, PICKERING 1997) and 64 % at Texas A&M University (CATO 1990). Some jars showed ethanol concentrations above 75 % although topping up usually was done with this concentration. The most likely explanation is the use of higher concentrated ethanol when filling the jar for the first time. Another possibility is a deviation of the measurement results from the "real" ethanol concentration, because substances, having leached from the specimens, increased the liquids density. A titration of the water concentration (method by Carl Fischer) can reveal such differences. NEUHAUS et al. (2012) showed that readings of a "Density Meter" may be 4–6 % (at most 8 %) higher than the result of such a titration.

Jar sizes and closure types

We present here, to our knowledge for the first time, a comprehensive investigation of the effect of jar size and closure type on ethanol concentration. Both effects, however, occasionally have been mentioned in the literature. PICKERING (1997) suggested that jar size and type of specimens do not affect ethanol concentration, thus supporting our results on jar sizes. PALMER (1996) also mentioned the jar size, analysing 272 containers from the Smithsonian Institution mammals' wet collection: "fluids in the largest containers exhibited […] lower ethanol concentrations […] than all other glass containers". She ascribed this to the fact that they "are filled with as many specimens as possible". This is quite plausible. The high water content inside fresh (or formalin fixed) specimens may lower the ethanol concentration over time, especially if initial preservation lacks placing them in a series of incrementing ethanol concentrations. This is especially pronounced in crammed large jars (see also the Alcomon presentation handout http://www.alcomon.com/steilcms/pdf/5.pdf). Another possibility for low concentrations in large jars may be an unfavourable ratio of the jar volume and the contact area between jar and its closure. Doubling the jar size roughly increases the contact area by the second power, but the volume by the third power. Therefore the closure of large jars may provide less resistance to the pressure exerted by the vapour, due to thermal expansion and contraction of the liquid. Crammed large jars are present in our collection as well, and may be the reason for lower mean ethanol concentrations in large jars of some collections, when compared to medium jars. Large ground glass stopper jars from the Myriopoda and Arachnoidea collections, as well as large screw cap jars from the Crustacea collection, presented such results (lower concentration in larger containers). They were, however, statistically insignificant (Fig. 6).

PALMER (1996) continues "no single glass style, despite kind of closure, was shown to be superior to any of the other glass styles". This agrees with our results of similar ethanol concentrations in jars of both closure types. Hence, our choice for jars, made of

standard glass with polypropylene screw caps (plus a low density polyethylene inlay), seemed to be ideal, given the comparably low price and the additional advantage of much easier manipulation. Palmer (1996) unfortunately did not elaborate on the glass styles and their duration of presence in the collection. In our collections, screw cap jars were introduced only a decade ago, and transfers from old jars to new jars regularly include an exchange of ethanol. For that reason we expected the new jars to hold significantly higher concentrations of ethanol, which was not the case. One explanation is that a considerable number of transfers to new jars proceeded without exchanging literally all the ethanol, e.g. when for conservation purposes the sump together with some old ethanol was transferred to the new jar. The more likely reason is that the ethanol was quite recently exchanged completely in a much higher number of old ground glass stopper jars than expected at the beginning of our study.

A prospective topic for our maintenance activities will be permeability and diffusion of gaseous substances and preservative fluids through polymers such as our screw caps. "In polyethylene, polypropylene, and Plexiglas™ jars, the whole surface is permeable to most preservative fluids, thus these containers have a larger area for diffusion compared to glass jars" (van Dam 2000). Measurements on the permeability of polymers for water, quoted in van Dam (2000), show that our screw caps (polypropylene – PP – caps and low density polyethylene – LDPE – inlays) are more permeable than other polymers, e.g. high density polyethylene (HDPE), polyethylene terephthalate (PET) and polyvinylidene chloride (PVDC). He also reports hardening and splitting of the plastics of low-priced, thin-walled PE and PP buckets purchased around 1970. Palmer (1996) warns "None of the non-glass containers, including fiberboard liquipaks, high density polyethylene buckets or tanks composed of either stainless steel, unidentified metals or wood, appear to be suitable as specimen storage containers." Although these reports refer to whole jars and containers made from polymers, they may apply in a minor extent to our polymer screw caps – at least such an effect was already indicated by Levi (1966). This calls for cautious observation of such jars and long-time comparison with other jar types.

Modern glass jars with polymer screw caps may also have a different sealing power than old jars with ground glass stoppers. Changing temperatures (in storage areas lacking climate control) cause the preservative fluid to expand or contract. This expansion is much more intense than that of glass, even violent enough to break jars (unless the seal gives way). We experienced that especially with large ground glass containers after moving them to our climate control area; van Dam (2000) reports such incidents as well: "For example, at 20 °C EtOH has an expansion coefficient that is approximately 40 times higher than borosilicate glass, whereas that of water is eight times higher than glass […]. Combined with the increased vapour pressure the seal will be under considerable stress […]". Our new jars with polymer screw caps have a rim of 2 mm for all jar sizes, whereas jars with ground glass stopper have a considerably larger rim of 8-25 mm, permitting for jar size. It is possible that the thin rim of the new jars, even with tightly closed screw caps, offers less resistance to ethanol vapour than the wide rim of ground glass stopper jars. Our results on small jars from the Mollusca collection seem to confirm this assumption. They were the only small jars under climate control and their ground glass stopper jars perform not only better than in all other collections, but also better than the screw cap jars next to them.

Refilling ethanol

Ethanol in a mixture with water evaporates – due to its higher vapour pressure – considerably easier, causing the ethanol concentration of the mixture to decrease. Theoretical data on that process and other properties of ethanol-water mixtures can be found in a particularly detailed paper by WALLER & STRANG (1996). Experimental data by ULRICH (1997) generally confirm them, at least for common concentrations in wet collections, since he concluded that ethanol at a concentration of 82 % or below always evaporates (at room temperature) as 82–83 % ethanol. The theoretical data (WALLER & STRANG 1996), however, indicate that mixtures between 60-75 % evaporate ethanol at an even higher concentration of 86–88 %, whereas mixtures below 20 % evaporate ethanol of less than 70 %. Relative humidity influences evaporation of water from the water-ethanol mixture and exceptionally high values will cause condensation of humidity into the mixture, diluting the ethanol (WALLER & STRANG 1996). On the other hand evaporation of a given substance, e.g. ethanol, does not depend on the presence of vapour from other substances (such as water), it only depends on temperature, surface area of the liquid, vapour pressure, and the amount of vapour (from the given substance) above the liquid.

Compensating this evaporation of ethanol – at high vapour concentrations – was not accomplished in our collections so far, because we filled up with 75 % ethanol, which was clearly insufficient. NEUHAUS et al. (2012) consider filling up with 85 % ethanol to be insufficient, and WALLER & SIMMONS (2003) noted that even using a 95 % v/v solution for topping up raises the mean ethanol concentration only a little. A short calculation confirms that: A typical 100 ml jar may contain 20 cm³ specimens and an ethanol concentration at the mean value from our results, which is 66 %. Topping up a loss of 10 % of the volume (= 10 ml) with 75 % ethanol would increase the concentration by 1 % to 67 %; with 85 % ethanol the increase would be 2 % and with 96 % ethanol (the concentration usually on sale) the increase would be 4 %, for a final concentration of 70 %. This is clearly below our target of 75 %. NOTTON (2010) provided detailed information on topping up, including a comprehensive table for selecting the appropriate ethanol concentration.

However, our jar definitely requires a replacement of a part of the ethanol to reach the target, not only topping up. Thus, reaching a sufficient ethanol concentration requires a measurement of concentration and a calculation of the necessary amount of ethanol to be replaced. For convenience, we performed our calculation in a way that avoids the preparation of various ethanol concentrations for topping up; instead we modify the amount of liquid to be replaced in a way that allows us to use 96 % ethanol only (Fig. 10). It is important to note that in our calculation the specimens supposedly are completely solid. The wide variety of body densities, especially among invertebrates, does not allow for a general correction. Additionally, considering the – unknown – amount of ethanol inside the specimens for topping up would sometimes result in ethanol concentrations inside the jar – but outside the animal – well above 75 %, which should be avoided because of the associated high osmotic stress (WALLER & STRANG 1996). In case of very low ethanol concentrations not all of the 96 % ethanol should be added at once, but in several steps to avoid extreme concentration changes. Filling up with 96 % ethanol includes the possibility to exceed the target ethanol concentration; therefore Fig. 10 also provides thresholds for using 96 % ethanol to refill jars, applicable for any lost volume.

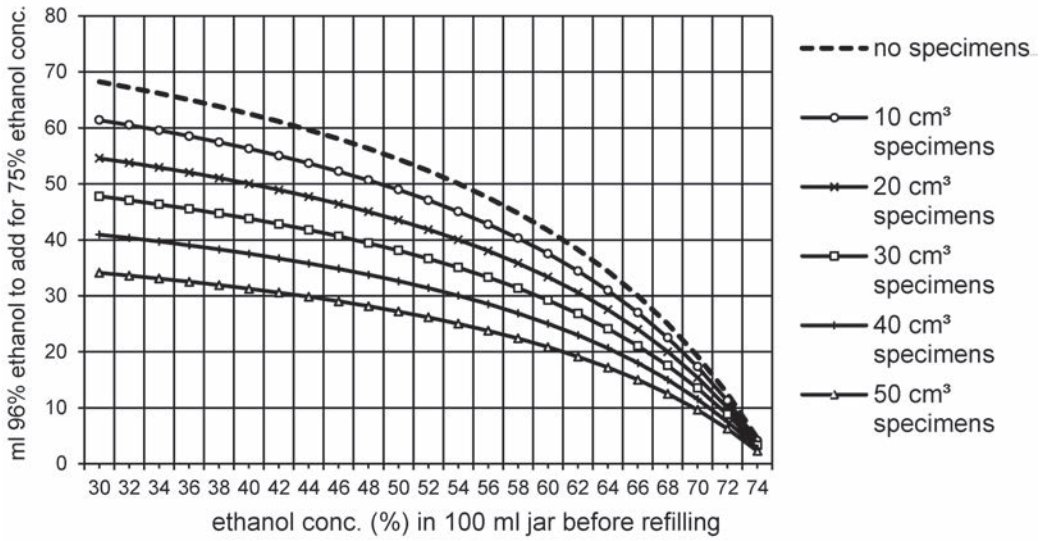


Fig. 10. This figure may be utilized in two ways. First to determine (for an 100 ml jar) the amount of liquid (y-axis) with a certain ethanol concentration (x-axis), that must be replaced by 96% ethanol to get an ethanol concentration of 75%. Note that the ethanol inside the specimens is not considered. This avoids concentrations above 75% in the jar and the resulting osmotic stress, but as water diffuses from the animal the concentration will fall. The bigger the specimens inside (cm³ specimens), the smaller the amount of liquid, that must be replaced. OR: At a certain amount (percentage from total volume) of missing ethanol in the jar (y-axis), there exists one threshold ethanol concentration that is allowed in it (x-axis) for staying at or below the target concentration of 75%, if topping up is done with 96% ethanol.

For example, a loss of 10% of the volume must be refilled (start at "10" on the y-axis to find the threshold). Using 96% ethanol for refilling will not exceed the target of 75% unless a jar with 50% v/v specimens (line for 50 cm³ specimens) already contains a threshold concentration of 70% ethanol; it would be 72% ethanol for jars with 20% v/v specimens, or 72.5% ethanol for 0% v/v specimens. Only if the evaporation loss is considerable and larger quantities must be refilled, then the use of 96% ethanol probably pushes the concentration above the 75% target (Fig. 10).

In order to reach optimal ethanol concentrations, changing our strategy from complete exchange of all ethanol to a partial exchange or filling up appears practicable. This is because, today, practical concentration measurement techniques are available (e.g. the "Density Meter"; or the Alcomon indicator system). Moreover, a partial exchange of ethanol also helps minimizing the loss of substances that have leached from the specimens into the storage fluid. Fatty acids, lipids, amino acids and structural proteins have been detected (von Endt 1994). Lipids and fatty acids should be reduced, since the latter "may denature and degrade tissues and decalcify bones" (Moore 2002), but usually leaching of amino acids and proteins is undesirable. Each complete ethanol exchange starts a new cycle of intense leaching, and a partial exchange of ethanol has the potential to keep that process at bay.

Possible reasons for low ethanol concentrations

Extremely low ethanol concentrations may result for several reasons:

(1) the jar may have been filled with ethanol of improper concentration in the first place (e.g. incoming acquisitions with too low concentration);

(2) increased evaporation due to a defective stopper / lid or insufficiently closed jars or due to frequent manipulation;

(3) filling up with ethanol of too low concentration to compensate for evaporation losses.

(4) a high water content inside fresh (or formalin fixed) specimens may lower the ethanol concentration over time, especially if initial preservation misses placing them in a series of incrementing ethanol concentrations.

The sample from the Myriopoda collection included six jars (= 10%) with ethanol concentrations between 46% and 50%, which can be attributed to the effect of refilling for decades with a too low concentration. The ages of the samples agree with that: they were donated at the end of the 19th century and around 1930, respectively. We believe that the intensive research in the Myriopoda collection might be the reason for the lower ethanol concentration in the screw cap jars compared to the other collections (Tab. 4).

Four jars from the Mollusca collection (7%) had ethanol concentrations between 37% and 48%, although all ethanol was exchanged 20 years ago, when the collection was moved to our climate control area. Clearly, their ground glass stoppers sealed imperfectly, allowing for a constant loss of ethanol vapour. This loss was not especially conspicuous because, otherwise, the jar would have been singled out for special treatment during our regular inspections.

The collection of Arachnoidea contains five jars (= 8%) with ethanol concentrations between 28% and 53%. Four of them contain scorpions (the fifth pseudoscorpions, a completely different taxon). The donation dates are 1883, 1925, and twice 1974. The low ethanol concentrations in the former two may be attributed to the effect of replenishing with 75% ethanol. The latter two are screw cap jars which were studied by the same person in the mid-1970s. It can be assumed that the ethanol was not replaced at all after handling or that its concentration was decreased on purpose to soften the animals.

The small number of jars with exceptionally low ethanol concentrations shows that a regular, but for each single jar extremely rare, exchange of all ethanol – implemented according to the experience of our maintenance staff – is very efficient. The much higher quota of jars with extremely low ethanol concentration among the Evertebrata Varia collection might have two explanations. Firstly, most of them contained tiny specimens or specimens with fragile structures. Therefore a complete ethanol exchange was avoided in these collections. Secondly, this part of the collection is the oldest of all wet collections and therefore was subjected to evaporation for a longer period.

Small Crustacea jars with ground glass stoppers have on average much lower ethanol concentrations than all other small jars with that closure. Only in this collection was Vaseline, used as sealant, removed in the 1980ies. The use of Vaseline for ground glass stopper jars was highly recommended by ARNDT (1943) after a six-year trial in which Vaseline reduced ethanol evaporation by an incredibly high factor of 360. Elsewhere, in contrast, this treatment proved to be counterproductive because the Vaseline becomes

granular or colloidal (Levi, 1966, Moore 2007). Such a reaction was also reported from silicone grease recommended by Levi (1966) and subsequently used in the 1970s (Neuhaus et al. 2012) and vacuum silicone grease (Moore 2007). The latter also reports Vaseline becoming granular or colloidal; he instead recommends "Paraffin soft white is physically similar to Vaseline and so far (up to 30 years) has shown none of the problems […]". However, possible long-term consequences and influences on the chemical composition of the preservative and, as a consequence, on the specimens are still unclear.

Climate control

Small jars with ground glass stoppers seem to perform much better than small jars with screw caps when both are under climate control, as can be seen from the results for the Mollusca collection (Fig. 6). Twenty years ago the ethanol in all jars was exchanged, on the occasion of moving them to the climate control area. Ten years ago, small screw cap jars were introduced and started to replace defective jars. They were refilled with fresh ethanol. Nevertheless, they contain significantly less ethanol today than the ground glass stopper jars. It seems that the small ground glass jars retain ethanol much better than small screw cap jars (see discussion above regarding jar sizes/closure types and their possible influence on the sealing power). Finally, small jars with ground glass stoppers perform clearly better under climate control than without it, and this effect is missing among screw cap jars.

A high mean concentration of 68.9% was measured in small screw cap jars from the Arachnoidea collection (no climate control). They were mainly introduced during a maintenance focus on Arachnoidea only four years ago. In that short period they lost as much ethanol as the ground glass stopper jars under climate control which were filled 20 years ago. Importantly, moving jars to the climate control area requires special care to avoid cracks due to abrupt temperature changes.

Quality of ground glass stopper jars and production date

In the Myriopoda collection we unintentionally selected almost exclusively the old type of small ground glass stopper jars, which additionally seem to have been unopened for decades. Their ethanol concentration was clearly higher than in the temporally heterogeneous samples of small ground glass stopper jars from the other collections, but could not compete with the Mollusca jars, which were stored under climate control (Fig. 6). The quality of the old type of ground glass stopper jars appears to be the best.

DNA analysis

Our PCR tests show that it is possible to isolate DNA sequences even from quite old samples of the wet collection, albeit the size of amplification products is in general short. Yet, the longest marker sequence (428 bp) was obtained not only from a quite young sample, but also from two quite old samples. Several of the samples with low ethanol concentration proved successful in the PCR tests. The current ethanol concentration is clearly not informative to predict PCR success because it fails to reveal anything about preparation history. We assume that samples that were well conserved shortly after death may yield at least short sequences even after long periods of time and even if the ethanol concentration decreased over time. Note, however, that the DNA analyses performed here

were intended as accessory tests to show whether old samples of the Evertebrata Varia wet collection are in principle suitable for DNA analyses. Clearly, broader investigations are needed to explore any possible correlation between storage, ethanol concentration and PCR success. The positive sample (Fasc3) with the high formaldehyde concentration indicates that further research is needed. As mentioned above, even this concentration of formaldehyde (400 mg/l) is low compared to the commonly used concentration of a 4 % v/v formaldehyde solution.

Recommendation

Any handling of specimens in the wet collection (either by guests or staff) should be combined with a mandatory ethanol concentration check before putting them back into storage, which must be in the responsibility of the staff.

During regular inspections a jar should be supplied with an Alcomon® indicator for 60 % ethanol concentration, a small pellet designed to swim if the ethanol concentration is below the threshold.

Topping up jars during regular inspections should be done with 96 % ethanol, except the lost volume exceeds 15 % or the jar contains more than 30 % v/v specimens.

Climate control should be extended to all collections. For the time being, in the climate control area small jars should be preferred to have ground glass stoppers than screw caps, since according to our data they perform significantly better.

Conclusion

Our results yield various options for further improvement. Most of them are related to handling activities. Skilled maintenance activities, which cannot be replaced by technical features, seem the key factor for a successful long-term conservation of our wet collections. Furthermore, climate control, which presently is available for only a part of our collections since the late 1980s, should be applied to all parts of the wet collections.

In general the following factors play key roles in the long-term preservation quality of wet collections: Initial fixation/preservation protocol, choice of container, quality of ethanol (no impurities/denaturants), climate control, handling protocols (maintenance, monitoring, use in research).

Scientific collections are part of the cultural heritage of a country and are consequently under legal protection of the Hague Convention for the Protection of Cultural Property (chapter I, article 1a), as enacted in Austria in 1964 (BGBL. 58/1964) and legally implemented in the current § 13 of the "Denkmalschutzgesetz (BGBL I Nr. 170/1999)" and in the "Kulturgüterschutzverordnung (BGBL II Nr. 51/2009)". Their crucial role for answering scientific questions was discussed repeatedly (e.g. SUAREZ & TSUTSUI 2004). The Naturhistorisches Museum Wien has the legal obligation to "keep the holdings observing up-to-date standards of museology, science, logistics, safety appliances, climate conditions, preservation and restoration" (BGBL II Nr. 399/2009: Museumsordnung für das Naturhistorische Museum). Aware of the collection's importance as a basis for various fundamental and applied research fields, the State reserves the right to withdraw

collections or parts of collections from federal museums to prevent damage (§ 5 (6) Museumsgesetz BGBl. I Nr. 14/2002). Thus, preserving the collection is a strong legal obligation, not only a scientific requirement, and is a task of major public interest.

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