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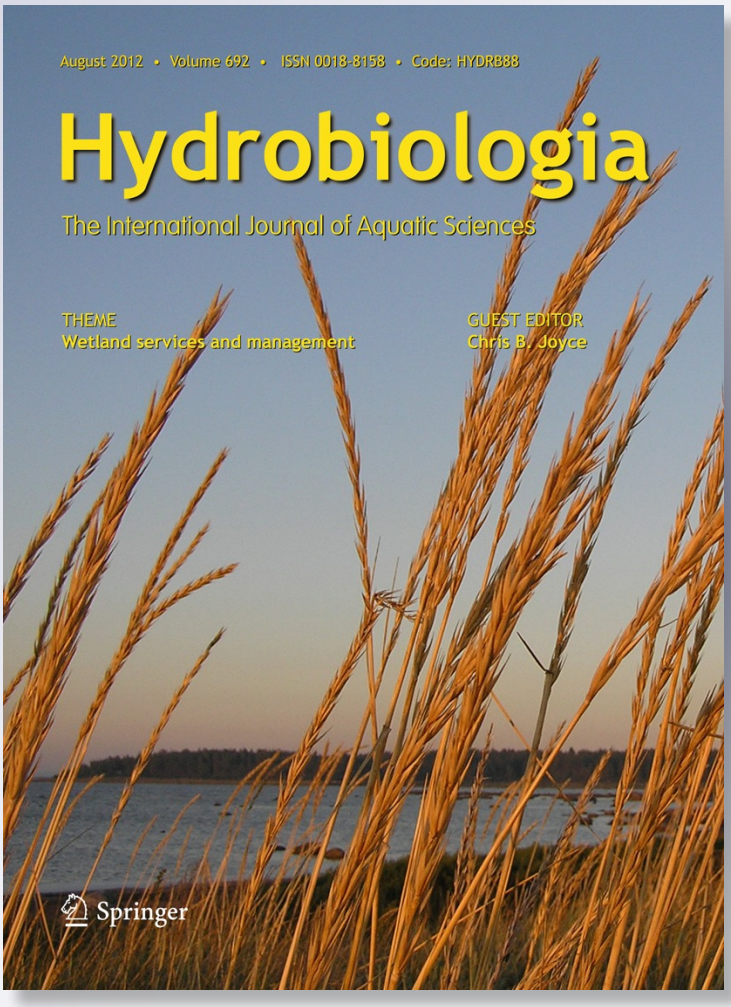

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ALGAE FOR MONITORING RIVERS

Identification versus counting protocols as sources of uncertainty in diatom-based ecological status assessments

Maria Kahlert · Martyn Kelly · Raino-Lars Albert · Salomé F. P. Almeida · Tomáš Bešta · Saúl Blanco · Michel Coste · Luc Denys · Luc Ector · Markéta Fránková · Daša Hlúbiková · Plamen Ivanov · Bryan Kennedy · Petr Marvan · Adrienne Mertens · Juha Miettinen · Joanna Picinska-Fałtynowicz · Juliette Rosebery · Elisabet Tornés · Sirje Vilbaste · Andrea Vogel

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Abstract In 2009, seventeen analysts participated in a pan-European diatom ring-test (intercalibration), analyzing nine samples from seven countries following the European standard EN 14407. The objective of this exercise was to agree on practical conventions on diatom identification to facilitate future intercalibration work and to assess the extent to which national differences in sample analysis (counting protocol and identification conventions) contribute to variability in EU-level comparisons of diatom-based methods. Differences in the reported taxa lists were large, but not a major source of variation in values of a common metric (the phytobenthos Intercalibration Common Metric, ICM). Therefore, every country can apply its own identification conventions for national assessments, and still be fairly confident that the ICM reflects the national classification of its streams. Part of the index variation was due to differences in counting protocols and care should be taken when handling broken valves, girdle views and small taxa. More work at both national and European level is needed to provide a harmonized way of using diatoms for ecological status assessments in the future.

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M. Kahlert (✉)
Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, P.O. Box 7050, 750 07 Uppsala, Sweden
e-mail: Maria.Kahlert@slu.se

M. Kelly
Bowburn Consultancy, 11 Monteigne Drive, Bowburn, Durham DH6 5QB, UK

R.-L. Albert · J. Miettinen
Ecomonitor Ltd., Länsikatu 15, 80110 Joensuu, Finland

S. F. P. Almeida
Department of Biology, GeoBioSciences, GeoTechnologies and GeoEngineering (GeoBioTec) Research Unit, University of Aveiro, 3810-193 Aveiro, Portugal

T. Bešta
Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic

S. Blanco
Department of Biodiversity and Environmental Management, University of León, 24071 León, Spain

M. Coste · J. Rosebery
Water Quality and Wastewater Department, Irstea, 50, Avenue de Verdun, Gazinet, 33612 Cestas Cedex, France

L. Denys
Research Institute for Nature and Forest (INBO), Kliniekstraat 25, 1070 Brussels, Belgium

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Introduction

'Macrophytes and phytobenthos' is one of the biological quality elements required for assessing ecological status of surface waters according to the European Water Framework Directive (WFD: The European Parliament and the Council of the European Union, 2000), the European legal framework for water protection. One of the key objectives of the WFD is for all water bodies to attain 'good ecological status' and the WFD also specifies the need for intercalibration of boundaries to ensure that 'good ecological status' represents the same level of ecological quality in all parts of Europe. This is important as a water body that does not attain good ecological status will require a potentially expensive 'program of measures' to attain this quality level.

In the first phase of the intercalibration exercise, 'macrophytes' and 'phytobenthos' were treated separately, with diatoms acting as a proxy for the latter. This first phase compared national assessment systems, definitions of conditions and the location of the high/good and good/moderate ecological status boundaries (Kelly et al., 2007, 2009) and resulted in the publication of legally-binding boundary values for those Member States involved (The Commission of the European Communities, 2008). During this exercise, however, data supplied by Member States were accepted at face value and the effect of national conventions on species identifications and of different national counting protocols were not addressed. Nevertheless, there is evidence that problematic groups of diatom taxa can add uncertainty to diatom analyses (Prygiel et al., 2002; Kahlert et al., 2009). So far, effects of national counting protocols on diatom counts and index calculations have not been reported. These problems need to be addressed to evaluate the results of the intercalibration obtained so far and to facilitate further work in the second and final phase of the intercalibration exercise.

To determine whether differences in diatom identification or national counting protocols are a major source of error when comparing ecological status class boundaries, seventeen trained diatom analysts from fifteen Member States participated in a pilot ring-test using their national counting protocol. All these protocols agreed with the European standard method for sample analysis (European Committee for Standardization, 2004). As well as indicating the scale of uncertainty due to taxonomic and other conventions, this exercise provided an opportunity to agree on practical levels of identification by which national datasets could be harmonized for further intercalibration work.

L. Ector · D. Hlúbiková
Department of Environment and Agro-Biotechnologies (EVA), Public Research Centre—Gabriel Lippmann, 41 rue du Brill, 4422 Belvaux, Luxembourg

M. Fránková
Department of Vegetation Ecology, Institute of Botany, AV ČR, Lidická 25/27, 602 00 Brno, Czech Republic

P. Ivanov
Faculty of Biology, Sofia University "St. Kliment Ohridski", 8 Dragan Tzankov Blvd., 1164 Sofia, Bulgaria

B. Kennedy
EPA, John Moore Road, Castlebar, Irl, Mayo, Ireland

P. Marvan
Limni s.r.o., Kalvodova 13, 616 00 Brno, Czech Republic

A. Mertens
Grontmij Nederland B.V., Division Water, Team Ecology, P.O. Box 95125, 1090 HC Amsterdam, The Netherlands

J. Picinska-Fałtynowicz
Department of Ecology, Institute of Meteorology and Water Management, National Research Institute, Wrocław Branch, Parkowa 30, A55 51-616 Wrocław, Poland

E. Tornés
Catalan Institute for Water Research (ICRA), H2O Building, Scientific and Technologic Park of the University of Girona, Emili Grahit 101, E-17003 Girona, Spain

S. Vilbaste
Center for Limnology, Estonian University of Life Sciences, Riia 181, 51014 Tartu, Estonia

A. Vogel
Inninger Str. 17, 82229 Seefeld-Hechendorf, Germany

A note on terminology: the most widely used term to describe an exercise of this nature is 'intercalibration'; however, this term is used in the WFD in a different context, namely the comparison of ecological status boundaries established by Member States. Our exercise was initiated to underpin this intercalibration exercise and it would be confusing to refer to this as an 'intercalibration'. Instead, we have opted for the term 'ring-test', which is widely used, informally, in the English-speaking world, to describe such exercises.

Our hypothesis was that removing major differences in taxa identification would significantly decrease the between-operator variation of the diatom index used for intercalibration work (Kelly et al., 2009). Considering the adherence to European standards, we did not expect a major influence of counting protocols. Although the primary focus of this exercise was to facilitate EU-wide comparisons of ecological status within the current intercalibration process, the results are relevant to many other situations where diatom monitoring is used to support decision making.

Materials and methods

Organization of ring-test exercise

Seventeen trained experts involved with diatom identification for monitoring purposes participated, representing Belgium Flanders (BE-FL), Bulgaria (BU), Czech Republic (CZ), Estonia (EE), Finland (FI), France (FR), Germany (DE), Ireland (IE), Luxembourg (LU), Poland (PL), Portugal (PT), Spain (ES), Sweden (SW), The Netherlands (NL) and United Kingdom (UK). A questionnaire amongst this group identified taxa complexes on which the study would focus.

Major problems were expected with the *Achnanthidium minutissimum* (Kützing) Czarnecki complex, the *Fragilaria capucina* Desmazières complex, the subspecific taxa of *Nitzschia palea* (Kützing) W. Smith and *Gomphonema parvulum* Kützing complex. Further points of concern were the *Cocconeis placentula* Ehrenberg complex and small naviculoids (biovolume <150 µm^3, e.g. *Eolimna*, *Fistulifera*, *Mayamaea*, and some *Sellaphora*). Numerous other taxa were also suggested but were considered less likely to influence index values significantly. These included the group of *Cymbella ventricosa* Kützing sensu lato complex (*Encyonema lange-bertalotii* Krammer, *E. minutum* (Hilse) D.G. Mann, *E. silesiacum* (Bleisch) D.G. Mann, *E. subminutum* Krammer, *E. ventricosum* (C. Agardh) Grunow), the *Gomphonema intricatum* Kützing complex (*G. pumilum* (Grunow) E. Reichardt & Lange-Bertalot/*G. minutum* (C. Agardh) C. Agardh complex), small *Nitzschia* (*N. frustulum* (Kützing) Grunow, *N. inconspicua* Grunow, *N. leistikowii* Lange-Bertalot, *N. liebetruthii* Rabenhorst) and the *Staurosira*/*Pseudostaurosira* group.

Participants were asked to provide samples containing taxa of the prioritized groups and to prepare slides for the ring-test. Slides were sent to the participants in spring 2009 for analysis by September 2009. All participants analyzed the slides according to their national conventions. They were also asked to photograph taxa that presented the greatest difficulties and to collect ecological information on them.

Results were collated and a workshop was held at the Centre de Recherche Public—Gabriel Lippmann in Belvaux, Luxembourg, 26–27 November 2009 for discussion and finding preliminary solutions for the most problematic taxa before initiating the second phase of the WFD intercalibration exercise.

Samples

All samples (Table 1) were collected from stones according to EN 13946 (European Committee for Standardization, 2003) between 2004 and 2008. Samples 2–8 were each collected from a single stream. Sample 1 was a pooled sample from three small rivers in Corsica (France) and sample 9 was pooled from four Polish samples of different ecological status. Three samples were from sites with rather high alkalinity (3, 6, 9), one from a river with high phosphorus (2), whereas three other streams had low phosphorus concentrations (5, 7, 8). The streams were situated at an altitude of 20–860 m a.s.l. and their catchment area ranged from 5 to 5,800 km^2. Most samples were from streams of high or good ecological quality with exception of sample 2 and one of the samples contributing to sample 9.

Slides from all samples were divided amongst the participants with the aim of ensuring their analysis by as many participants as possible despite resource limitations. Analysis of three samples was obligatory for all participants (1, 3 and 8). In addition, half the participants analyzed samples 2 and 7 as obligatory,

Table 1 Samples included in the first European ring-test exercise

Sample	River	Country	TP (mg l^{-1})	pH	Alkalinity (mmol l^{-1})	Conductivity (mS m^{-1})	N-NO_3 (mg l^{-1})	IPS	TI
1	Fango, Gravone, Cavo	France (Corsica)	<0.1[a]	7.5[a]	0.2[a]	9.9	1.0	17.6	1.4
2	Paimionjoki	Finland	0.2	7.5	0.7	12.8	1.2	10.2	3.0
3	Valmar Stream (Mondego Basin)	Portugal	0.1	7.7	5.5	56.3	0.13	16.4	2.1
4	Fonte Cova Stream (Lis Basin)	Portugal	0.1	6.7	0.1	12	1.1	17.5	1.5
5	Rio Ceira (Mondego Basin)	Portugal	0.02	7.8	0.2	4.2	0.16	18.0	1.5
6	River Coquet	UK	<0.02[b]	8.0	1.1	No data	No data	15.7	2.3
7	Tributary of River Aherlew	Ireland	0.002[b]	7.3	0.6	No data	No data	17.9	1.2
8	Ådalsån	Sweden	0.01[b]	6.8	0.3	3.5	0.01	16.9	1.1
9	Kamienna, Skrzynczanka, Wisła, Bóbr	Poland	0.04[a]	7.3[a]	1.1[a]	13.9	0.91	16.8	1.6

Chemistry given for annual means if available, otherwise spot values. IPS (CEMAGREF, 1982) and TI (Rott et al., 1999) calculated as median value for all ring-test participants

[a] Average of pooled streams

[b] SRP

whilst the others analyzed samples 5 and 6. Samples 4 and 9 were optional because their composition was similar to some of the other samples. Six participants analyzed all samples and five analyzed more than the required number of five. All samples were analyzed by 12 or more participants, except for 4 and 9 which were still analyzed by at least seven people.

Slides were of differing quality and valve density; some had significant numbers of broken valves or detritus in the sample, but all analysts had to deal equally with these common difficulties in diatom identification and counting. Slide 1, in particular, was rated as "very dense" by many participants (i.e., with more than 25 valves per field of view). Slides 4 and 5 also had a quite high cell density (16–25 valves per field of view). Some difficulties in enumeration and identification due to poorly visible valves could be expected. In addition, problems were encountered on slides 1 (fragmented valves), 2 (heterogeneous material, detritus) and slide 8 (detritus), possibly also contributing to variation in results (see also Appendix—Supplementary Material). Resources were too limited to investigate the effects of slide quality on the results.

Counting protocols and national conventions

The European standard for diatom sample analysis outlines general procedures but still allows several options regarding the counting procedure. It is, for example, possible to count either valves or frustules as a diatom unit. The number of units to be counted is given as '300–500 units but lower and higher numbers may be appropriate for some purposes'. There are also several possibilities for treating broken diatoms. A broken valve or frustule can be excluded, counted only if ¾ of the frustule is present or if at least one pole and the central area are present. If judged appropriate, apical fragments can also be counted as ½ valve. The emphasis is on a consistent approach to these issues within a monitoring program rather than on absolute conformity to a prescriptive method.

The European standard also allows for different approaches for enumerating valves observed in girdle view and separated raphid or araphid valves of heterovalvar taxa which can be identified only by examination of both valves. These options include direct identification (if possible), 'matching' to identifiable valve views, and otherwise identification at the lowest possible level. Differences between national counting protocols are summarized in Table 2.

Diatom identification conventions

Participants were asked to specify the literature used for diatom species identification. They were also asked to state their practical or national conventions to overcome difficulties in particularly problematic

Table 2 Summary of European counting protocols

Country	Counted diatom unit	Nr. counted	Broken valves	Girdles & separated raphid or araphid valves
BE-FL	Valve	500	Pole & centre, apical fragments = ½	Direct, match, %
BU	Valve	≥400	Pole & centre	Direct, %
CZ	Valve	≥500	Pole & centre apical fragments = ½	Direct, match, genus
DE	Valve & frustule	≥400	Pole & centre	Direct, match, genus, %
EE	Valve	≥400	Pole & centre	Direct, match, genus
ES	Valve & frustule	≥400	¾	Direct/match, otherwise not counted
FI	Valve	≥400	Pole & centre	Direct, match, genus
FR	Valve & frustule	≥400	¾	Counted if typical for a species
IE	Valve	>300[a]	Excluded	Direct, match, genus, %
LU	Valve	~400	¾	Direct, match, genus
NL	Valve	200 (400)	¾, pole & centre, apical fragments = ½	Mostly counted, direct
PL	Valve	300–400	Excluded	Direct, match, genus, %
PT	Valve	~400	Pole & centre	Direct, match, %[b]
SW	Valve	≥400	Excluded	Direct, match, genus, %
UK	Valve	>300[a]	Excluded	Direct, match, genus, %

Broken valves: excluded or counted if stated parts are visible. Girdles & separated raphid or araphid valves: 'direct' = visual identification if possible, 'match' = matching to identifiable valve views, 'genus' = identification to the lowest possible level, '%' = valves are attributed to identifiable valve views *prorata*

[a] Excluding planktonic taxa

[b] Only Cocconeis placentula agg

groups in routine monitoring. All analysts reported use of the second edition of the Süßwasserflora von Mitteleuropa (SWF) (Krammer & Lange-Bertalot, 1986, 2004a, b, 2007). Many also referred to the available volumes of *Diatoms of Europe* (DoE) (Krammer, 2000, 2002, 2003; Lange-Bertalot, 2001), *Bibliotheca Diatomologica* (BDia) (Krammer, 1997a, b) and also *Iconographia Diatomologica* Vol. 2 (Ico 2) (Lange-Bertalot & Metzeltin, 1996), plus original literature (Table 3). Most countries have adopted practical national rules to overcome difficulties in particularly problematic groups (Table 4).

Data collation

The resulting taxa lists, counts, national standards, conventions and counting protocols were collected by the Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences. Indices were calculated using an Access database. To facilitate comparisons, all data were converted to relative abundance of the total count and nomenclature was harmonized by changing synonyms, with all participants given the opportunity to assess changes. Non-Metric Multidimensional Scaling (NMDS) and cluster analysis, using the similarity measure Bray–Curtis (Kelly, 2001), followed by a Multi-Response Permutation Procedure (MRPP) were used to assess the similarity between and within samples and participants (PC-ORD Version 5.32, McCune & Mefford, 2006).

Calculation of the European diatom Intercalibration Common Metric (ICM)

The data were used to calculate the phytobenthos ICM, which was developed to compare status class boundaries during the first phase of the WFD intercalibration exercise (Kelly et al., 2009). The ICM is a combination of two widely used diatom metrics, the IPS (Specific Pollution Sensitivity Index/ Indice de Polluosensibilité Spécifique, CEMAGREF, 1982) and the TI (Trophic Index/Trophieindex, Rott et al., 1999). The IPS is often considered to assess 'general water quality' with low values indicating high pressure levels, whereas the TI is a trophic index

Table 3 Literature used for diatom identification

Country	SWF	DoE	Ico 2	Bdia	Other literature
BE	x	x	x	x	X[a, b, and more, also specific publications]
BU	x	x	x	x	
CZ	x	x	x	x	x[b]
DE	x	x	x	x	x[a, b, and more, also specific publications]
EE	x	x		x	x[c]
ES	x	x			
FI	x	x	x	x	x[a]
FR	x	x	x	x	x[b, and more, also specific publications]
IE	x		x		x[d, e]
LU	x	x	x	x	x[a, b, e, and more, also specific publications]
NL	x	x	x	x	x[b, and more, also specific publications]
PL	x	x	x	x	x[specific publications]
PT	x	x			x[b, e, also specific publications]
SE	x	x	x	x	x[a, b, and more]
UK	x	x		x	x[d, e]

[a] Alles et al. (1991)
[b] Diatom Research (Biopress Limited)
[c] Hustedt (1927–1966)
[d] Hartley et al. (1996)
[e] Prygiel & Coste (2000)

where low values indicate low nutrient concentration. Indicator values for each diatom taxon were derived from the software OMNIDIA 5.3 (Lecointe et al., 1993, http://omnidia.free.fr/). Both IPS and TI results for each sample and participant were converted into Ecological Quality Ratios (EQRs), which is the ratio between the observed value of the index and the expected value. Expected values were the mean values of reference sites used in the first phase of the WFD intercalibration exercise or, for Member States that did not take part in this, values set by an equivalent process (see Kelly et al., 2009). The ICM is the mean of the EQRs of the IPS and TI (Kelly et al., 2009) and was calculated as follows:

$$\mathrm{ICM} = \frac{\frac{\mathrm{IPS}}{\mathrm{IPS_{ref}}} + \frac{(4-\mathrm{TI})}{(4-\mathrm{TI_{ref}})}}{2}$$

Analysis of variance (ANOVA) followed by Tukey HSD tests for unequal N (Statistica Version 10, StatSoft, Inc., 2011) were used to test for differences of the ICM values between streams.

"What if" scenarios: new calculation of index values after merging taxa

The results were discussed at a workshop at the Centre de Recherche Public—Gabriel Lippmann in Belvaux, Luxembourg, 26–27 November 2009 which aimed to find practical solutions for harmonizing the identification of problematic diatom taxa ahead of the second phase of the WFD intercalibration exercise. The participants tried to identify those taxa which could be the cause for a Bray–Curtis similarity lower than 60% between participants within the same sample, a threshold over which analyses are generally considered to be replicates (Engelberg, 1987; Kelly, 2001). The participants discussed also the national convention protocols handling such problematic taxa. Problematic taxa were then merged and their index values averaged to overcome observed identification problems and to ensure that different degrees of 'splitting' or 'lumping' would not influence comparisons with the ICM.

Using this 'streamlined' harmonized taxa list, modified versions of both constituent metrics (IPS and TI) and the ICM were calculated and compared with the original values to see if our initial hypothesis of less ICM variation with a harmonized taxa list was confirmed. This comparison was done by testing for a significant difference of the ICM residuals (absolute difference of ICM value from median ICM value) before and after streamlining with ANOVA followed by Tukey HSD tests for unequal N (Statistica Version 10, StatSoft, Inc., 2011). It should be emphasized that the purpose of this exercise was to enable comparison of the taxa lists and ICM values between Member States for the second phase of the European WFD

Table 4 National conventions to handle problematic diatom taxa groups for routine monitoring (separate or merge varieties/species)

Country	ADMI	FCAP	CPLA	GPUM	NPAL	CVEN	GPAR
BE-FL	Separate	Separate	Separate	Separate	Separate	Separate	Separate
BU	Separate	Separate	Separate	Separate	Separate	Separate	Separate[a]
CZ	Separate[b]	Separate[b]	Separate[b]	Separate[b]	Separate[b]	Separate[b]	Separate[b]
DE	Separate	Separate	Separate	Merge	Separate	Separate[k,l]	Separate
EE	Merge	Separate[d]	Merge	Merge	Merge	Separate[c]	Separate[d]
ES	Merge	Separate	Separate	Merge	Merge	Separate[c]	Separate
FI[e]	Separate[f]	Separate[d]	Merge	Merge	Separate	Separate	Separate[d]
FR	Separate	Separate	Separate	Separate	Separate	Separate	Separate
IE	Merge	Separate[g]	Separate	Merge	Separate[h]	Separate[c]	Separate
LU	Separate	Separate	Separate	Separate	Separate	Separate[i]	Separate
NL	Separate[j]	Separate	Merge	Separate	Separate[h]	Separate[k]	Separate
PL	Separate	Separate	Separate	Separate	Separate	Separate	Separate
PT	Merge	Separate	Separate	Merge	Merge	Separate[c]	Separate
SE	Separate[f]	Separate[d]	Merge	Merge	Separate	Separate	Separate[d]
UK	Merge	Separate	Separate	Merge	Separate	Separate	Separate

ADMI, *Achnanthidium minutissimum*; FCAP, *Fragilaria capucina*; CPLA, *Cocconeis placentula*; GPUM, *Gomphonema pumilum*; NPAL, *Nitzschia palea*; CVEN, *Cymbella ventricosa*; GPAR, *Gomphonema parvulum*

[a] Does only separate *Gomphonema parvulum* f. *saprophilum* Lange-Bertalot & E. Reichardt, i.e. *G. parvulum* and *G. exilissimum* (Grunow) Lange-Bertalot & E. Reichardt are merged

[b] Both separation and merging are possible entries

[c] Separate *Cymbella minuta* Hilse in Rabenhorst from *C. silesiaca* Bleisch in Rabenhorst

[d] Problematic varieties separated by size according to Swedish method

[e] Laboratory routine, not a national method

[f] Varieties merged, but separated by size according to Swedish method

[g] *Fragilaria gracilis* and *F. rumpens* (Kützing) G.W.F. Carlson merged

[h] Separate *Nitzschia palea* var. *palea* from *N. palea* var. *debilis* (Kützing) Grunow

[i] Separate *Encyonema lange-bertalotii*, *E. minutum*, *E. silesiacum*, *E. ventricosum*

[j] Separate certain varieties

[k] Separate *E. minutum*, *E. silesiacum*, *E. subminutum*

[l] More varieties will be separated in future

intercalibration exercise, and not aimed at simplifying or changing any national diatom methods.

Assessment of sources of variation other than taxa identification

The next step in our study was to evaluate additional sources of variation (other than taxa identification) in both taxa counts and ICM results, and to explore if some of these should receive further attention in forthcoming studies. One source of variation examined was differences in the national counting protocols. Other sources which could not be addressed here in more detail due to the nature of this study were variation in diatom slide preparation or the error within single slides (Prygiel et al., 2002; Alverson et al., 2003; Lavoie et al., 2005; Blanco et al., 2008).

Results

ICM calculation

The ICM was significantly different between samples (ANOVA, $P < 0.001$), reflecting the ecological differences between the streams. The overall differences between participants were quite low for all but samples 1 and 4 (Fig. 1). The ICM residuals (absolute

Table 5 List of changes to taxa lists recommended by workshop participants for phase II diatom intercalibration

Taxa	Action	Revised IPS sensitivity	Revised TI sensitivity
All taxa identified only to genus	Omit		
Achnanthidium minutissimum sensu lato (including *A. catenatum*)	Merge	5.0	1.2
Amphora inariensis, *A. pediculus*	Merge	4.0	2.8
Asterionella spp.	Omit		
Cocconeis placentula	Merge varieties	3.9	2.1
Cyclostephanos spp.	Omit		
Cymbella ventricosa complex (*E. lange-bertalotii*, *E. minutum*, *E. silesiacum*, *E. ventricosum*, etc.)	Merge	4.9	2.0
Fragilaria capucina complex excluding *F. vaucheriae* (see below)	Merge	4.2	1.3
Fragilaria crotonensis	Omit		
Fragilaria vaucheriae sensu lato (*F. capitellata*, *F. vaucheriae*)	Merge	3.4	1.8
Gomphonema intricatum complex (*G. angustum*, *G. minutum*, *G. pumilum* including varieties)	Merge	5.0	1.4
Mayamaea/*Fistulifera*	Merge	2.2	3.0
Nitzschia palea	Merge varieties	1.0	3.0
Pseudostaurosira/*Staurosira*	Merge both genera	3.8	2.8
Stephanodiscus spp.	Omit		
Thalassiosira spp.	Omit		

Indicator values (V) for the merged taxa for the IPS are all set to "1"

Indicator values for the merged taxa for the TI are unchanged

difference of ICM value from median ICM value) were not significantly different between samples 2, 3, 5–9 (ANOVA, Tukey HSD for unequal N, $P > 0.05$) but were significantly higher for samples 1 and 4 (ANOVA, Tukey HSD for unequal N, $P < 0.05$).

Diatom taxa composition

A total of 701 taxa names were used in the nine samples. Accounting for obvious synonyms [e.g. *Fragilaria ulna* (Nitzsch) Lange-Bertalot, *Synedra ulna* (Nitzsch) Ehrenberg, *Ulnaria ulna* (Nitzsch) Compère] reduced this number to 546 of which 203 (37%) were represented by single records and 303 (56%) never attained a relative abundance >1%. 166 taxa (30%) remained below both these benchmarks.

Inspection of differences in taxa richness gave a first hint of differences in national counting protocols and possible causes of the ICM differences. On average, analysts encountered 21–48 taxa per sample, with sample 2 tending to be the richest in taxa. However, there were quite large differences per sample: taxa numbers recorded by different analysts for sample 2 ranged for example from 37 to 92. Part of the higher taxa number was caused by different conventions in the way taxa were handled (Table 4). For example, some analysts pooled *Cocconeis placentula* varieties into one taxon only; others separated a number of varieties or species and used the name *C. placentula* only for *C. placentula* var. *placentula* sensu stricto (Aboal et al., 2003; Monnier et al., 2007; Jahn et al., 2009). Another example for this was the *Gomphonema intricatum* complex, which was by some identified using the Süßwasserflora concept resulting in one taxon (e.g. *G. pumilum* following Krammer & Lange-Bertalot, 2004b, Table 85, Figs. 13–19), or was separated into different varieties respectively species (*G. angustum* C. Agardh, *G. minutum*, *G. pumilum* including varieties; Reichardt, 1997). Both the *C. placentula* varieties and the *G. intricatum* complex were therefore considered candidates for a merging when comparing European diatom taxa lists or ICM values.

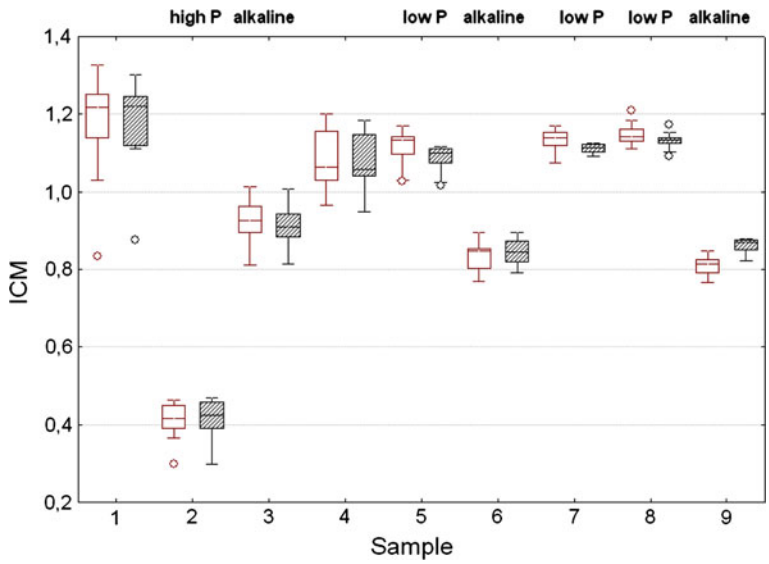

Fig. 1 Variation of diatom index ICM (European diatom ICM) on original diatom data (*open boxes*) and 'streamlined' data (*shaded boxes*) for the nine slides analyzed in the ring-test. 'Streamlining' implied the merging of taxa with relative abundances <2%, and using merged taxa pools with averaged index values (see Table 5). *Boxes* interquartile range (25–75%), *lines* non-outlier range, *circles* outliers

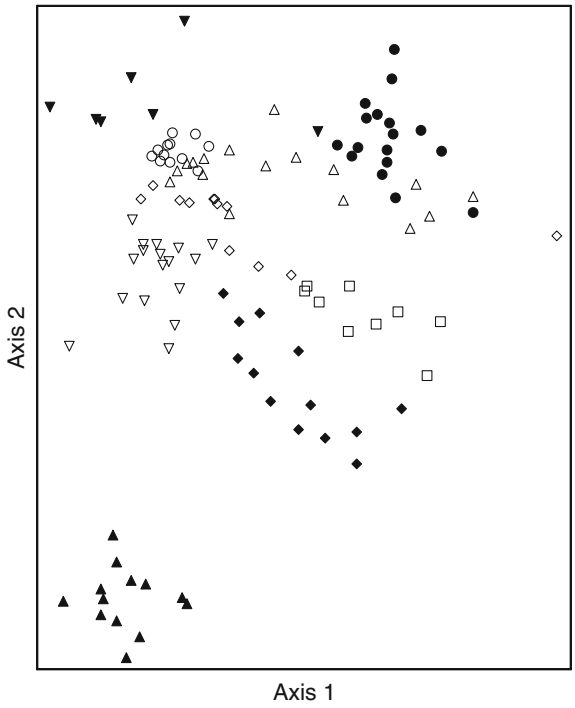

Fig. 2 Similarity between the nine slides counted by 17 analysts for the European ring-test (original results). *Open triangle* sample 1, *filled triangle* 2, *open inverted triangle* 3, *filled inverted triangle* 4, *open diamond* 5, *filled diamond* 6, *open circle* 7, *filled circle* 8, *open square* 9. Within-group variation less than between-group variation (NMDS, similarity measure Bray–Curtis, stress 13.4, MRPP $P < 0.001$; PC-ORD 5.32)

Inspecting the diatom taxa lists for samples yielding low similarity values was the next step to identify sources of variation between ICM index values. In general, diatom community composition was more similar within samples than between samples (MRPP, PCOrd version 5.32, distance measure Bray–Curtis with the nine samples as groups, chance-corrected within-group agreement $A = 0.36$, $P < 0.001$), and no analyst was consistently different from the others (Fig. 2). Samples 2 (with the highest phosphorous content) and 4 and 8 (with the lowest pH values) were relatively separated by the NMDS from the rest of the samples. The other samples were found in the middle of the diagram but still well separated from each other. However, even if most of the analysts grouped together for each sample, the Bray–Curtis similarity between certain analysts was in many cases below <60% (Figs. 2, 3). We now looked for taxa complexes which were abundant in a sample, included species or varieties which were treated differently by different participants and which, in some cases, had different sensitivity values for IPS or TI, thus influencing the ICM value.

In the case of sample 1, which had the largest differences in the ICM, about half of the participants identified *Achnanthidium catenatum* (J. Bílý & Marvan) Lange-Bertalot, whereas the others recorded this as representative of the *A. minutissimum* complex. As the sample was dominated by these two taxa, and *A. catenatum* has a relatively low sensitivity value in the IPS compared to other representatives of the *A. minutissimum* complex reported from this sample [*A. minutissimum* f. *inconspicuum* (Østrup) Compère & Riaux-Gobin, *A. microcephalum* Kützing, *A. caledonicum*

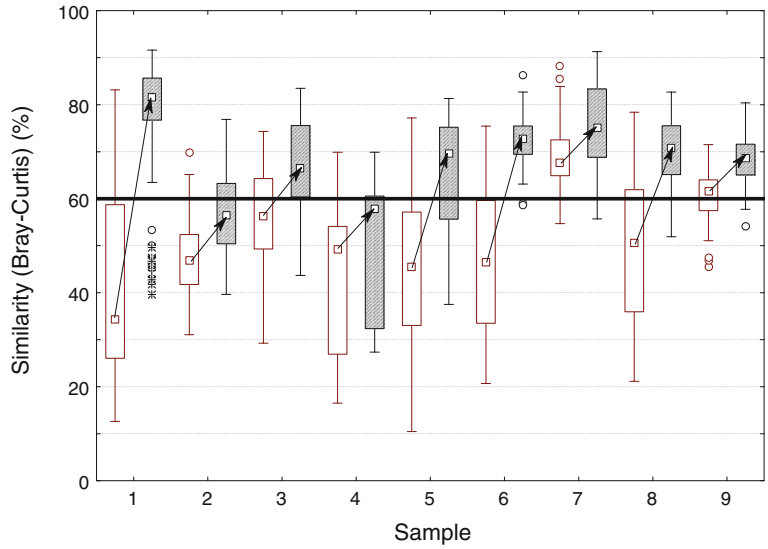

Fig. 3 Variation of within-sample similarity (measure: Bray–Curtis) when comparing raw diatom data (*open boxes*) and 'streamlined' data (*shaded boxes*) for the nine slides analyzed in the ring-test. 'Streamlining' implied the merging of taxa with relative abundances <2%, and using merged taxa pools with averaged index values (see Table 5). *Boxes* interquartile range (25–75%), *lines* non-outlier range, *circles* outliers. *Black line* 60% similarity, threshold over which samples could be considered to be replicates (Kelly, 2001)

(Lange-Bertalot) Lange-Bertalot and *A. subatomus* (Hustedt) Lange-Bertalot], differences in identification had a quite large effect on the IPS value. Depending on the proportion of *A. catenatum* recorded, differences in the IPS were on average 3 units on a scale from 1 to 20, which changed the classification of a stream from high IPS value (average: 18.8) to lower quality (average IPS 15.1). The consensus of the workshop participants was that *A. catenatum* was present in this sample. Other taxa recorded frequently in this sample were the *Gomphonema intricatum* group, *Ulnaria ulna* and *Navicula notha* J.H. Wallace.

Sample 4, the other sample with high variation in the ICM, was characterized by one (or possibly several) small naviculoids, which were identified very differently by participants, and occasionally not even counted. Participants who identified this as a "pollution-tolerant" taxon [*Eolimna minima* (Grunow) Lange-Bertalot, *Navicula submuralis* Hustedt, *Sellaphora seminulum* (Grunow) D.G. Mann, *Mayamaea recondita* (Hustedt) Lange-Bertalot] had lower IPS values than those who chose a taxon lacking IPS index values (*Naviculadicta* species, *Eolimna rhombelliptica* Gerd Moser, Lange-Bertalot & Metzeltin), or did not record the taxon at all. Sample 4 also contained a relatively high proportion of *Eunotia* species (reflecting the low pH value), and a relatively high proportion of *Karayevia oblongella* (Østrup) M. Aboal. Other taxa characteristic of this sample were the *Fragilaria capucina* complex, *Psammothidium subatomoides* (Hustedt) Bukhtiyarova & Round and *Placoneis* sp.

Sample 2, from the river with the highest phosphorus content, was most clearly separated from the other samples in the NMDS. It was characterized by the centric diatom *Melosira varians* C. Agardh along with a number of small naviculoids and *Nitzschia* species. Differences in identification were apparent for the *Nitzschia* taxa, which were identified by some as taxa relatively sensitive to eutrophication and organic pollution [e.g. *N. acidoclinata* Lange-Bertalot, *N. hantzschiana* Rabenhorst, *N. perminuta* (Grunow) Peragallo], whereas others identified only taxa typical for impacted waters [e.g. *N. supralitorea* Lange-Bertalot, *N. liebetruthii*, *N. subacicularis* Hustedt, *N. pusilla* (Kützing) Grunow).

In sample 3, another taxa complex was handled differently amongst participants and contained a mix of *Amphora pediculus* (Kützing) Grunow, *A. inariensis* Krammer and *Caloneis* species along with the *Achnanthidium minutissimum* complex, small naviculoids and small *Nitzschia* species. Some analysts did not note *Amphora inariensis* at all, whereas others observed very different proportions of this species. This is related to the problematic distinction of *A.*

pediculus and *A. inariensis* in Krammer & Lange-Bertalot (1986).

In sample 5, identification of different representatives from the *Cymbella ventricosa* complex may have caused differences in index values, as the taxa named by different analysts had different sensitivity values, or no index values at all (in which case they were omitted from calculations). Most participants identified the taxon as *Encyonema silesiacum*, which is assumed by the IPS to be the most sensitive taxon in the complex, and consequently increased ICM values. Others identified *E. ventricosum* or *E. minutum*, both of which have lower sensitivity values for the IPS. The rest of the mentioned taxa lack index values (*E. silesiacum* var. *latum* Krammer, *E. silesiacum* var. *altense* Krammer, *E. minutiforme* Krammer) and were, consequently, omitted from IPS calculations. The other important taxa group in this sample was the *Achnanthidium minutissimum* complex.

In sample 7, also characterized by a high abundance of the *A. minutissimum* complex, obvious differences in taxa lists were due to different identification of the *Gomphonema intricatum* complex as well as taxa related to *G. olivaceoides* Hustedt. Participants encountered different amounts of *G. angustivalva* E. Reichardt, *G. pumilum* var. *rigidum* E. Reichardt & Lange-Bertalot, *G.* aff. *minusculum* Krasske, *G. pumilum*, *G. micropumilum* E. Reichardt, *Gomphonema* spp., *Gomphonemopsis exigua* (Kützing) Medlin, *Gomphonema pumilum* var. *elegans* E. Reichardt & Lange-Bertalot, *G. calcifugum* Lange-Bertalot & E. Reichardt, *G. designatum* E. Reichardt, *G. lateripunctatum* E. Reichardt & Lange-Bertalot, *G. minutum*, *G. olivaceoides* and *G. olivaceum* (Hornemann) Brébisson. Other characteristic taxa of sample 7 included *Karayevia oblongella* and *Fragilaria gracilis* Østrup.

In samples 8 and 9, different approaches to the *Fragilaria capucina* complex were apparent, especially the attribution to *F. gracilis*, *F. rumpens* (Kützing) G.W.F. Carlson and *F. vaucheriae* (Kützing) J.B. Petersen differed between participants. In sample 8, the IPS was, for example, negatively correlated with the proportion of *F. rumpens* but positively with the proportion of *F. gracilis*. Samples 8 and 9 were otherwise quite different from each other. Sample 8 was characterized by a low pH and a high proportion of *Eunotia* species, furthermore *K. oblongella*, the *A. minutissimum* complex, *Tabellaria flocculosa* (Roth) Kützing and some *Rossithidium* sp. Sample 9 contained the *A. minutissimum* complex, *Diatoma mesodon* Kützing and *Gomphonema parvulum*.

Sample 6 was separated from the other samples by a high abundance of the *C. placentula* group which was identified differently by the participants. Also here, the recorded taxa have different index values causing part of the ICM variation. The sample also contained *Nitzschia fonticola* (Grunow) Grunow, *Reimeria* sp. and the *A. minutissimum* and *F. capucina* complexes.

"What if" scenarios: new calculation of index values after merging taxa

The next step after discussion at the Luxembourg workshop was to merge problematic taxa groups and to average their index values. Using this new preliminary 'streamlined taxa' list, modified versions of both constituent metrics (IPS and TI) and the ICM were calculated and compared with the original results.

Recalculating the similarity with the 'streamlined taxa list' generally increased Bray–Curtis similarities, and most samples had then median values above the 60% threshold (Fig. 3). The 'streamlined ICM' was also closely correlated to the original ICM (Pearson correlation coefficient, $r = 0.994$; Fig. 4). However,

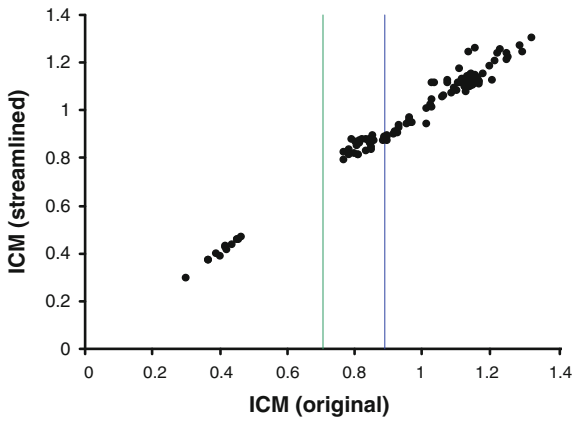

Fig. 4 ICM (European diatom ICM) calculated on 'streamlined' diatom data is linear correlated to ICM calculated on raw data in the ring-test. 'Streamlining' implied the merging of taxa with relative abundances <2%, and using merged taxa pools with averaged index values (see Table 5). *Lines* show ecological status class boundaries (mean of acceptable band) decided on the Central/Baltic GIG Phytobenthos Intercalibration Exercise (good/moderate: 0.71; high/good: 0.89; Kelly et al., 2007)

variation in the ICM did not decrease significantly for any of the samples (Figs. 1, 4; ANOVA on ICM residuals, $P > 0.05$). The remaining unsolved identification problems only concerned a low number of counted valves. This strongly suggested that identification is only one of a number of factors contributing to between-count variation.

Other factors contributing to variation in diatom counts

The next step in our study was to present a list of possible alternative sources for the variation in both taxa counts and ICM results, and to perform some pilot analyses to find out if some of these factors should receive further attention in the future. As stated earlier, we focused in this study on national conventions on species identification and counting protocols. Other sources of variation, such as the random variation for repeated counts or the variation due to slide preparation were not addressed here. Table 2 describes differences in counting conventions between participants. There are a total of 256 possible combinations of these factors and we have not attempted to disentangle all of these. Instead, two examples are presented below to illustrate possible effects of the national counting protocols.

Inconsistency of counting broken valves

The effect of different handling of broken valves on the variability between participants is demonstrated by the example of long *Fragilaria* taxa in the samples 1 and 8. In effect, these reflect differences in what analysts regard as a 'record' of an individual in their counts. Included in the 'long *Fragilaria* taxa' were taxa identified as *F. capucina* var. *capucina* Desmazières, *F. capucina* var. *capitellata* (Grunow) Lange-Bertalot, *F. capucina* var. *perminuta* (Grunow) Lange-Bertalot, *F. gracilis*, *F. rumpens*, *F. tenera* (W. Smith) Lange-Bertalot, with some *F. biceps* (Kützing) Lange-Bertalot, *F. alpestris* Krasske, *F. capucina* var. *austriaca* (Grunow) Lange-Bertalot, *Synedra rumpens* var. *scotica* Grunow, *F. nanana* Lange-Bertalot, *F. nanoides* Lange-Bertalot, *F. famelica* (Kützing) Lange-Bertalot, *Ulnaria ulna*, and *Synedra*. Indeed, there was a tendency, albeit not statistically significant, for those who excluded broken valves to have lower relative abundances of long *Fragilaria* in sample 8 (Fig. 5B) and of *U. ulna* in sample 1 (Fig. 5A). As an example, higher amounts of *U. ulna* in sample 1 were connected to lower ICM values as *U. ulna* has relatively low index values. However, as the effect of counting broken valves differently was quite variable and not consistent, care must be taken when interpreting these data.

Inconsistency of counting girdle views and diatom units

It was also clear that two participants had relatively low counts of *Achnanthidium* sp. sensu lato and small naviculoids. After consultation, it became clear that these analysts did not record the girdle views of such taxa. Most analysts included all girdle views and tried to identify these to the lowest possible level adopting various methods (Table 2). However, some stated that they count girdle views 'only if they were typical for a

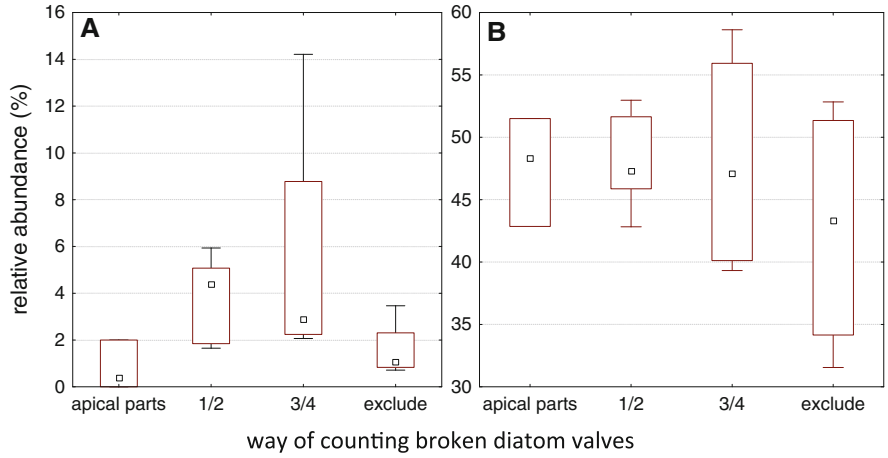

Fig. 5 Counted relative abundance (%) for diatom taxa *Ulnaria ulna* (**A**) respective long *Fragilaria* (**B**) when counting broken valves in different ways: apical parts: even apical pieces are counted ($n = 3$), 1/2: counted when one pole and center remain ($n = 6$), 3/4: counted only when ¾ of valve remains ($n = 4$), exclude: fragmented valves excluded ($n = 4$)

species' and otherwise not at all, which should lead to lower numbers for certain taxa. Moreover, the way that girdle views are treated is often characteristic for each taxon and it is not always easy to unravel the impact on the data. A low number of small taxa can also be the result of overlooking them in a dense slide or from counting two valves of a small frustule as one diatom unit instead of separate valves.

Discussion

The hypothesis that the removal of major differences in taxa identification would lead to a significant decrease of the ICM variation, the diatom index used for WFD intercalibration, has to be rejected as the 'streamlined taxa list' had little effect on within-slide variation. Other sources of variation must be considered, one of which is differences in national counting protocols, which we originally did not expect to play a major role. The European Standard (European Committee for Standardization, 2004) is written in broad terms and there is considerable scope for national variations within this. However, despite all the differences recorded between individual counts, the ICM (and the two constituent metrics) were robust enough to reflect the differences between the studied streams well, and differences between analysts were less than the differences between samples. Even though participants had different approaches to taxonomy (Tables 3, 4) and different counting protocols (Table 2), conclusions were generally comparable to the extent that the advice given to water managers would have been similar.

It could be argued that this ring-test represents a 'worst-case-scenario' as it focused on the most problematic taxa. In practice, these taxa complexes are widespread and the problems we address have also been noted in previous exercises (Morales, 2002, 2004; Prygiel et al., 2002; Lavoie et al., 2005; Kahlert et al., 2009) and relate back to incomplete knowledge of the taxonomy of these groups and imprecise descriptions of differential characteristics in the literature (examples: *Achnanthidium catenatum*, *Fragilaria gracilis*/*F. rumpens*, *Amphora pediculus*/*A. inariensis*; the recently published descriptions of the *A. minutissimum* complex (Ector, 2011) and *Amphora* sp. (Levkov, 2009) were not available at the time of the ring-test and are therefore not considered here). What this study has shown is that the error introduced by taxonomy has little effect on the calculation of diatom indices and the resulting interpretation of ecological status. Diatom indices are remarkably robust against inter-analyst taxonomy, probably due to the relatively large amount of diatom units counted for each analysis. The more diatoms you count, the closer you get to a stable mean of their relative abundances (The Law of Large Numbers, Stevenson et al., 2010). A closer focus on analytical procedure might be more beneficial in the future. This is not to say that identification is never a major source of uncertainty for the calculation of diatom indices (Prygiel et al., 2002; Kahlert et al., 2009) and even more so when using diatom indicators derived from cluster analyses and ordinations (Stevenson et al., 2010) .

It was not possible in this exercise to disentangle how much of the variation between analysts was due to random error and how much to systematic error introduced by differences in analytical procedures adopted by different countries. Further studies are needed to unravel the effects of different handling of counted diatom unit, the minimum number of units to count, and strategies for dealing with broken valves and girdle views (Blanco et al., 2008). Our study has shown that Member States should be able to compare metric values and assessments of ecological status, although compiling and sharing raw count information between Member States is likely to be subject to significant systematic errors even after taxonomic conventions have been harmonized.

Biogeography introduced an additional complication as many of the samples included taxa that were unfamiliar to some of the participants. This ring-test gives a good example of the differences in sample counting throughout Europe, but the variation in taxa identification may have been lower had analysts only encountered floras with which they were familiar. It is important that ring-tests are performed at a regional, as well as at a European scale. The former helps to train and harmonize analysts working in the same geographical region or political unit whilst the latter compares different national counting protocols and identification conventions, ensuring comparability at the European level. Such ring-tests also facilitate the transfer of knowledge, particularly with respect to taxa such as *Achnanthidium subhudsonis* (Hustedt) H. Kobayasi whose distribution seems to be changing (Coste & Ector, 2000), or *Psammothidium abundans*

(Manguin) Bukhtiyarova & Round, once overlooked but recently shown as common in the whole Nordic region (Van de Vijver et al., 2008).

Our workshop should be the start of a more long-term harmonization work similar to that performed in North America (United States Geological Survey and Phycology Section Patrick Center for Environmental Research, NAWQA Workshops, available on internet at http://diatom.acnatsci.org/nawqa/Workshops.aspx). Macrozoobenthos ring-tests have shown that training and intercalibration exercises have benefits both in improving the taxonomic knowledge of participants and the performance of metrics (Haase et al., 2006).

Regarding the original purpose of this study, i.e., to ensure the comparability of diatom taxa lists in the WFD intercalibration, we have positive results. The 'streamlined' ICM gave similar results to the original ICM. Every country can apply its own identification conventions for national assessments, and still be fairly confident that the ICM reflects the national classification of its streams. The streamlined ICM will provide a more robust basis for future intercalibration work, particularly those aspects that require direct comparison of taxonomic information. It is important to emphasize that this list is not a recommendation for national assessments of ecological status where a more detailed knowledge of a regional diatom flora may well inform ecological status assessments. Ring-tests of macrozoobenthos have also shown that merging of problematic taxa decreases variation (Buffagni & Furse, 2006), but this approach is a pragmatic one and it is possible that information may be lost (Verdonschot, 2006). In this instance, the streamlined diatom taxa list allows the key ecological information to be extracted so that we can proceed with intercalibration.

Conclusion

Although our ring-test revealed some problems when comparing diatom counts from diatomists in several European countries, the general picture is a positive one: diatom indices reflect prevailing water quality, despite variations in identification conventions, counting protocols, and probably slide quality. This, in turn, means that we can intercalibrate to a common standard. In practical terms, the ICM, as the mean of two metrics based on weighted averaging of indicator values, should be insensitive to minor variations in relative abundance and composition. Merging 'difficult' taxa into groups on a level at which all participants agree has little effect on the ICM although, at the same time, it does not seem to reduce within-sample variability of the diatom metric either. The use of a 'streamlined taxa list' returns similar ICM values as the use of the original taxa counts, which makes it possible to compare the streamlined diatoms taxa lists of different European countries directly. However, the source of the remaining variation of the ICM (and, by extension, other metrics) is still unknown, and should receive further attention in the future. In particular, we urge caution when raw data from several countries are compiled and compared.

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