

Original Research Article

DOI - 10.26479/2017.0303.13

DATA QUALITY IN ECOLOGICAL STATUS ASSESSMENT BASED ON DIATOM COMMUNITIES

Cristina Martone¹, Claudia Vendetti², Camilla Puccinelli², Stefania Balzamo¹, Sabrina Barbizzi¹,
Stefania Marcheggiani², Gioia Benedettini³, Laura Mancini²

1. Institute for Environmental Protection and Research, ISPRA – CN LAB Unit
– Via di Castel Romano 100 – 00128 Rome, Italy

2. Italian National Institute of Health, ISS – Viale Regina Elena 299 - 00161 Roma, Italy

3. Tuscany Regional Agency for Environmental Protection ARPAT – Area Vasta Costa - Via Marradi 114 -
57126 Livorno, Italy

ABSTRACT: From the emanation of Water Frame Directive 60/2000/EC, the assessment of ecological quality of water environment is entrusted to biological elements. The methods developed on biotic communities should be comparable and reproducible. In this context, a first interlaboratory comparison, also namely proficiency test, organized by ISPRA (Institute for Environmental Protection and Research) and ISS (Italian National Institute of Health) was performed with the participation of 27 operators of the Regional Environmental Agencies (ARPA/APPA). The exercise focused on taxonomic identification and counting of diatoms, applying the Intercalibration Common Metrics index, ICMi. The assessment of inter and intra-operator variability was carried out and the z-score was calculated in order to assess operators' "performance". The results show that the main sources of variability are diatom taxonomy and counting protocols. The statistical elaboration and the evaluation of z-score shown that most of the operators results are comparable. As a consequence, this study highlights the need for the future to regularly organize both teaching opportunities and further meetings and learning events and demonstrates the importance of implementing the use of reference material collections to allow quality controls and progress in diatom identification. In this paper we also reported an example of how to assess the quality of data aimed at the classification of water body by estimating the variability of the operator. This specific study was carried out by a small number of experienced traders operating from the same Italian region.

KEYWORDS: diatom, proficiency test, Intercalibration Common Metric index

***Corresponding Author: Dr. Cristina Martone Ph.D.**

Institute for Environmental Protection and Research, ISPRA – CN LAB Unit – Via di Castel
Romano 100 – 00128 Rome, Italy

* Email Address: cristina.martone@isprambiente.it

1.INTRODUCTION

The European Union water policy, the Water Framework Directive 60/2000/EC (WFD) [1] states that all European surface water bodies have to be classified according to their ecological status. The implementation of this Directive (in Italy, Legislative Decree 152/2006 [2] and subsequent decrees [3]), ensures an ecological approach oriented to sustainable development and integrated management of water resources. The Directive defines the ecological quality status as an expression of the quality of the structure and functioning of biological elements associated with surface waters. The biological elements point out for monitoring of rivers are: phytoplankton, macrophytes and phytobenthos, benthic invertebrate fauna and fish. The ecological status classification is based on the analysis of biological communities expressed in term of composition and abundance of species. Those data come from the monitoring activities results. As proxies for the phytobenthos were chosen diatom communities: they represent the most abundant and diverse group of algae of these benthic organisms [4,5]. Diatoms are unicellular algae in the class of Bacillariophyceae and are widely used for evaluating general water quality, monitoring rivers [6, 7, 8] and lakes [9,10,11] and for investigating more specific events such as eutrophication and acidification [12,13]. WFD requires that monitoring results and ecological data from aquatic environments are of a known and verifiable quality (Annex V of WFD). This request drives the Regional Environmental Agencies (ARPA/APPA) involved in Italian monitoring to ensure that the data produced from laboratory and field analyses are comparable. The quality assurance required that the monitoring results are fit-for-purpose. Interlaboratory comparisons are a valuable quality assurance tool for measurement laboratories since they allow direct monitoring of the comparability of testing results. Proficiency tests are interlaboratory comparisons that are organized on a continuing or ongoing basis and is becoming an integral feature of laboratory accreditation. The results generated in proficiency testing are used for the purpose of continuing assessment of the technical competence of operators involved in monitoring. For Diatoms, the skill of the operators in the taxonomic identification and valve counting can have a significant influence on the reliability and accuracy of the classification [14]. If diatom sampling is easier than other biological element the most difficult aspect is the identification at species level of these microscopic algae, based on morphological analysis of frustules. In Italy a first proficiency test organized by ISPRA (Istitute for Environmental Protection and Research) and ISS (Italian National Institute of Health) was performed and was focused on taxonomic identification and counting of

diatoms, since these steps represent the main source of variability associated with the ecological status assessment, applying the Intercalibration Common Metrics index, ICMi [15]. In this study the assessment of inter and intra-operator variability was measured by means the z-score in order to assess operators' "performance" in the taxonomic identification and counting of diatoms, with regard to their reproducibility (showing the degree of correlation between measurement results when the individual measurements are carried out under varying conditions) and (repeatability) intermediate precision (showing the degree of correlation between repeated measurements when the individual measurements are carried out under similar conditions). The evaluation of the parameters mentioned above is a fundamental part of the process to ensure the quality of monitoring data. The goal of this study was a harmonization of diatom identification and counting among operators and aims at estimating the reproducibility associated with this phase of the method. Through the analysis of proficiency testing results, it is possible to verify the critical aspects associated with the identification of the diatoms, as well as to assess the ability of operators to apply the biological method.

2. MATERIALS AND METHODS

Sampling and preparation of reference material

Sampling was performed at River Farfa, a left tributary of Tiber river. A group of experts selected the site where to perform sampling of benthic diatoms, following standard procedure [16,17]. They gathered 5 superficial pebbles of approximately 25 cm² each, over a total area of 100 cm². This sample was then oxidized in the laboratory by using hot hydrogen peroxide and slides were prepared by using Naphrax, a special resin having a high degree of refraction. Reference slides were prepared by applying all the official procedures of ISPRA method; analyses in labs were performed in compliance with UNI EN ISO 17025 and ISO 9001:2008. The permanent slides were collected as a reference material. All permanent slides were prepared from a unique dilution of the oxidized sample; the reference group of experts has then followed all the necessary steps to identify diatoms at species level and count diatoms valve on each slide. Permanent slides were analysed by previously marked them with a numerical identifying code. The origin of samples stayed unknown, in order to ensure operators' impartiality in slide analysis. To carry out the exercise, each operator was provided with a permanent slide where to perform the counting and calculation of the ICMi.

Diatom identification and counting

Identification of diatoms is based on feature identified at species level by a morphological identification of the cell wall. Diatom cell wall, frustule, is made of silica, composed by an upper and a lower valve. The surface of valves is composed of several ornaments, called striae, formed from rows of puncta, alveolae or similar structures. The shape of the diatom frustule and its ornamentations are species specific. Identification is performed using a light microscope with 1000x magnification, and image software analysis to detect measures of valves, length, width and number of striae in 10µm. Identifications were performed using iconographic guides [18; 19; 20; 21;22; 23;24 25, 26].

Calculating the ICMi

The ecological status was assessed by the calculation of the Intercalibration Common Metrics Index, ICMi, the Italian national method for the ecological status assessment based on diatom communities. The Ecological status classification of a given water body is presented as a deviation of the biological community from the same biological element but in reference condition expressed by the Ecological Quality Ratio EQR. The Ecological Status (ES) was classified into 5 quality classes of increasing degradation, from Bad to High, based on a value that represents the deviation from the least disturbed conditions. ICMi was developed in the European Inter-calibration Process, and it is composed by the Indice de Polluo-sensibilité Spécifique (IPS) and the Trophic Index (TI) [28, 29] and obtained by calculating the arithmetic average resulting from the Ecological Quality Ratio (Ecological Quality Ratio, RQE) of the two indexes IPS and TI.

$$ICMi = \frac{EQR_{IPS} + EQR_{TI}}{2}$$

$$EQR_{IPS} = \frac{\text{Observed value}}{\text{Reference value}}$$

$$EQR_{TI} = \frac{(4 - \text{Observed value})}{(4 - \text{Reference value})}$$

Indice de Polluo-sensibilité Spécifique (IPS) and the Trophic Index (TI) are two of the biotic index based on diatom communities. Each of indices takes into account the sensitivity of each species to different pollution sources and a confidence value as biological indicator. In particularly The IPS accounts for general quality estimates, TI measures mainly nutrient load [30]. The values of the two indexes are calculated through the formula of Zelinka & Marvan [31]:

$$IPS_5 = \frac{\sum_{j=1}^n a_j \cdot IPS_{I_j} \cdot IPS_{S_j}}{\sum_{j=1}^n a_j \cdot IPS_{I_j}}$$

$$IPS = (4,75 \cdot IPS_5 - 3,75)$$

$$TI = \frac{\sum_{j=1}^n a_j \cdot TI_{G_j} \cdot TI_{TW_j}}{\sum_{j=1}^n a_j \cdot TI_{G_j}}$$

where

a_j = abundance of valves of species j in sample;

S_j= sensitivity values vary from 5 (very sensitive) to 1 (very tolerant);

I_j= indicator values (tolerance) vary from 1 to 3;

TW_j= tolerance of the species to the nutrient concentration (0,1-4);

G_j= indicative weight of j species (1-5).

Coefficients of each index are proper of each diatom species (IPS_I, IPS_S, TI_G, TI_TW) and reported in Mancini & Sollazzo [15].

Proficiency test (PT)

The proficiency test was performed following ISO/IEC 17043:2010 [32]. The scheme adopted during the test was described in a protocol distributed to participants, the key points of the protocol were:

- determination of the assigned value associated with each reference slide;
- calculation of the performance statistics;
- evaluation of performance.

z-score is calculated as [3]:

$$z = \frac{X_{OP} - X_{Ref}}{\sigma} \quad \text{Eq.1}$$

Where:

X_{OP} is the mean value over all slides associated with each operator;

X_{Ref} is the reference value (assigned value obtained from the experts);

σ is the standard deviation for proficiency assessment: standard deviation associate with the assigned value.

A z-score above 3 or below -3 means that the participant result shall be considered to give an “action signal”. Likewise, a z-score above 2 or below -2, shall be considered to give a “warning signal”. The proficiency test followed two consecutive phases: 1) observation and identification of species on selected and fixed visual fields of some reference slides: positive results obtained in this phase of the analysis were determined by the correct identification of at least 80% species in a selected visual field; 2) counting and calculating the ICMi following the remarks listed in the official procedure.

3. RESULTS AND DISCUSSION

Sampling and preparation of reference material

River Farfa, with calcareous geology, is included in Italian Mediterranean region and can be classified as M4, one of the river macrotypes reported in Ministerial Decree 260/2010 [3]. Physical-chemical parameters detected are: pH (7.23), temperature (16.6 °C), and conductivity (530 μS/cm²). 32 permanent slides were prepared.

Diatom identification

27 operators among 32 participants of this PT overcame the first phase, identifying more than 80 % of diatom species in the selected visual field.

A total of 133 diatom species and varieties have been identified during the analysis of the 27

operators, 40 of them have been recognized only by one operator (Tab. 4). Most representative species of this sample were: *Achnantheidium minutissimum*, *Fragilaria ulna*, *Nitzschia dissipata*, *Nitzschia palea*, *Gomphonema olivaceum*, *Navicula cryptotenella*, *Nitzschia capitellata*, *Surirella brebissoni*, *Cylotella meneghiniana*, *Diatoma moniliformis*, *Encyonema minutum*.

Species as *Achnantheidium minutissimum*, *Fragilaria ulna*, *Nitzschia dissipata*, are recognized by all of operators; *Nitzschia palea*, *Gomphonema olivaceum* and *Navicula cryptotenella*, *Nitzschia capitellata*, *Surirella brebissoni* are identified by more than the 80 % of operators. On average 31 species were found by each operator, with a maximum of 43 taxa and the minimum of 22 (Fig. 1).

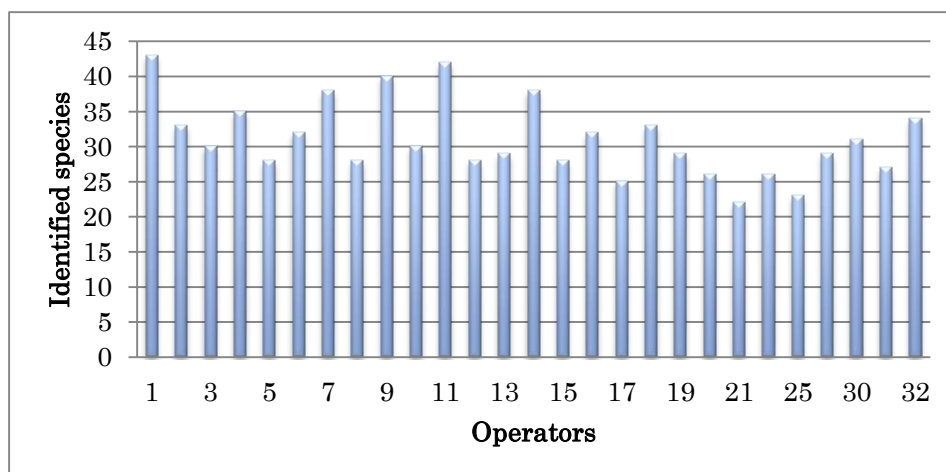


Figure 1. Number of species identified by each operator

Proficiency test results

Statistical analysis

In Table 2 are reported the results associated with the participants/operators: the mean value, the standard deviation and the standard deviation of the mean value, as well as the corresponding CV%, (coefficient of variation). The assigned values were evaluated as the mean value from the results obtained by the three experts for each slide. Furthermore, bias % for each operator was calculated in respect to the corresponding assigned value and in the last column is reported bias by operators in respect to the grand mean value calculated over all the assigned values (27) of the experts.

Table 2 – Values of the ICMi calculated by participants-operators, assigned values and basic statistics

Slide / operator	ICMi calculated by operators	ICMi assigned values	Standard deviation	Standard deviation of the mean	CV %	Bias (% ICMi operators vs assigned value)	Bias (% ICMi operators vs grand mean)
1	0,27	0,24	0,019	0,011	7,8	8,6	12,3
2	0,27	0,26	0,028	0,016	10,8	3,5	12,4
3	0,20	0,24	0,041	0,023	16,7	-19,0	-16,8
4	0,23	0,23	0,004	0,002	1,7	0,3	-2,3
5	0,23	0,21	0,013	0,008	6,2	8,5	-3,6
6	0,23	0,24	0,004	0,002	1,7	-1,9	-2,0
7	0,25	0,24	0,003	0,002	1,3	2,3	5,6
8	0,20	0,24	0,005	0,003	1,9	-16,4	-16,8
9	0,30	0,24	0,023	0,013	9,4	24,3	26,4
10	0,24	0,24	0,009	0,005	3,6	1,1	2,0
11	0,24	0,23	0,008	0,004	3,3	6,8	3,1
12	0,22	0,23	0,017	0,010	7,3	-6,8	-8,7
13	0,27	0,24	0,040	0,023	16,3	9,6	13,0
14	0,35	0,24	0,006	0,004	2,6	45,3	47,3
15	0,23	0,23	0,004	0,002	1,6	0,2	-3,6
16	0,25	0,24	0,014	0,008	5,8	2,2	3,6
17	0,30	0,24	0,061	0,035	25,3	24,7	26,8
18	0,37	0,30	0,043	0,025	14,2	20,9	54,1
19	0,34	0,25	0,074	0,043	29,3	35,3	45,3
20	0,31	0,26	0,060	0,035	23,1	19,0	30,9
21	0,35	0,22	0,004	0,002	1,7	55,4	47,0
24	0,29	0,22	0,008	0,005	3,7	31,9	22,2
25	0,22	0,25	0,029	0,017	11,6	-10,4	-6,2
29	0,26	0,24	0,020	0,012	8,3	4,3	7,7
30	0,13	0,21	0,013	0,008	6,4	-36,7	-44,2
31	0,28	0,23	0,022	0,013	9,7	26,1	20,3
32	0,23	0,22	0,002	0,002	1,0	2,9	-2,2

Considering ICMi values, on the basis of statistical analysis such as Shapiro-Wilk normality test and Grubbs test, the value associated with slide n. 18 corresponding to the expert operator and the value associated with the slide n. 30 corresponding to the participant/operator were “outliers”. So, these values were rejected.

Afterwards, it was applied basic statistics in order to determine respectively the grand mean assigned value and the associated standard deviation (ICMi assigned value = 0.24 ± 0.01) and the grand mean consensus value on participants' results with the associated standard deviation (ICMi consensus value = 0.27 ± 0.05).

In Figure 2a are reported z-score values associated with each participant/operator. Z-scores were calculated as defined in the equation 1 where σ is the standard deviation of the assigned value reported above.

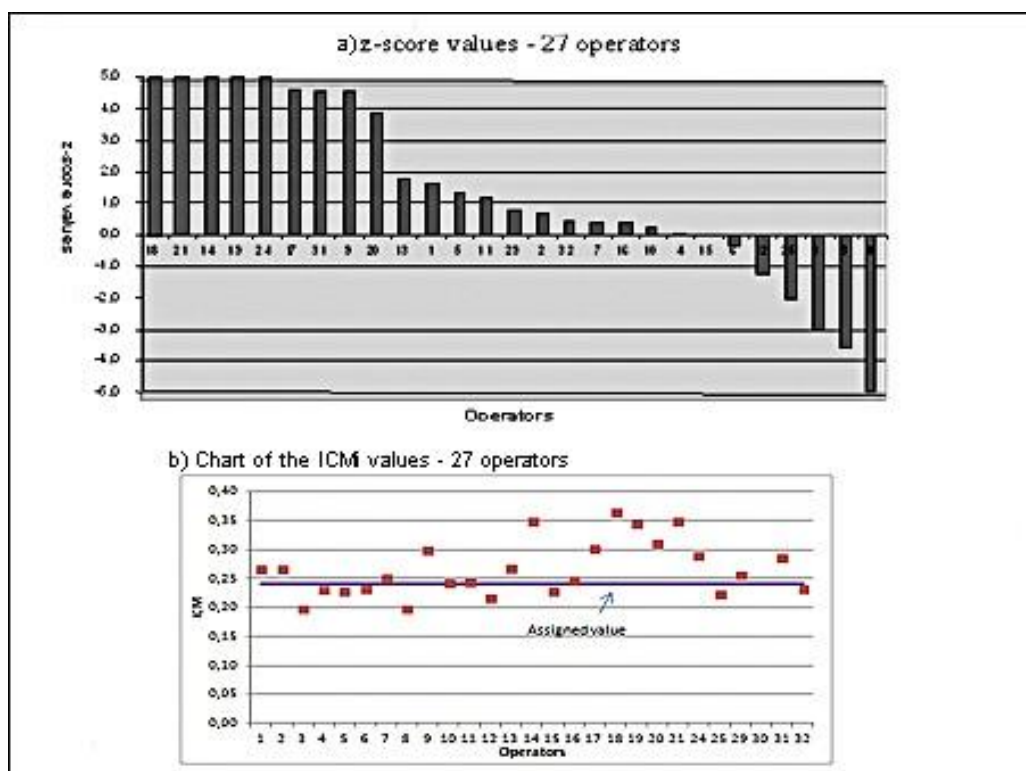


Figure 2. a) Z-score values by 27 operators; b) Results obtained from the 27 operators: chart of the ICMi values, horizontal row identify the reference value (continuous row).

Figure 2b graphically presents the results of participants, where the average value represents the reference value of the ICMi parameter, against which the comparison is made in order to assess the performance of operators for the PT and described as follows: every operator is associated with a symbol (square). The horizontal row correspond to the assigned value.

Case study: In order to evaluate repeatability (intermediate precision, showing the degree of correlation between repeated measurements when the individual measurements are carried out under similar conditions), 8 reference slides randomly selected among the 32 used for the PT were counted and calculated the ICMi by a group of 9 operators different of those participated to PT. Each slides was read 5 times by each operator. ANOVA test was performed and results obtained (total counts, average, standard deviation, CV%) are reported in tab 3 (operator n.5 was excluded).

Tab 3 – Case study

	<i>Operator</i> <i>1</i>	<i>Operator</i> <i>2</i>	<i>Operator</i> <i>3</i>	<i>Operator</i> <i>4</i>	<i>Operator</i> <i>6</i>	<i>Operator</i> <i>7</i>	<i>Operator</i> <i>8</i>	<i>Operator</i> <i>9</i>
Total counts	40	40	40	40	40	40	40	40
Average	0,21	0,25	0,23	0,25	0,22	0,23	0,22	0,23
Variance	2,24E-04	1,06E-03	1,73E-04	1,84E-04	2,81E-04	1,28E-04	1,74E-04	1,27E-04
Standard								
deviation	0,01	0,03	0,01	0,01	0,02	0,01	0,01	0,01
CV%	7	13	6	6	8	5	6	5

4. CONCLUSION

The analysis of biological elements includes several sources of uncertainty: some related to environmental factors, but others are related to analytical factors. In particular, identification and valve counting are considered the main sources of uncertainty in the water quality evaluation based on the analysis of Diatom [33,34]. The differences between the diatom species lists and consequently variations of ICMi values can be related mainly to following factors: diatom taxonomy classification and different counting protocol followed by operators. In this proficiency test dominant species were correctly identified: *Achnanthis minutissimum*, *Diatoma moniliformis*, *Fragilaria ulna*, *Gomphonema olivaceum*, *Navicula cryptotenella*, *Nitzschia dissipata*, *Nitzschia capitellata*, *Nitzschia palea*, *Surirella brebissoni*. Also species morphologically similar, *N. palea* and *N. capitellata*, have been recognized in most cases (Tab. 1). One source of uncertainty was due to identification guides used by operators: it was not recommended to participants which iconographic guide should be used, and each operators used theirs guides. Diatom taxonomy has been revisited in last three decades, from Krammer *et al* 86-2000 to Lange Bertalot 2000-2003 where most of Naviculaceae were divided in new genera. The valves identified as *Cylotella meneghiniana* and *Cylotella kuetzingiana* probably referred to the same species; in those slides where the one of them was recognized the other was absent: in Krammer *et al*, [20] *Cylotella kuetzingiana* is a synonym of *Cylotella meneghiniana*. Another difference in species is related to following species of Genus Cymbella. This genus has been revisited from 1980. Cymbella sensu lato, *Cymbella minuta*, *Encyonema minutum*, *Encyonema ventricosum*. Genus Cymbella has been divided in three genera: *Cymbella*, *Encyonema*, *Encyonopsis*, *Encyonema minutum* and *Encyonema ventricosum* are the current accepted names and synonyms, respectively of *Cymbella minuta* and *Cymbella ventricosum*. In Krammer *et al* [18] reported *Cymbella minuta* and *Cymbella ventricosa* (old name of *E. ventricosum*) as synonyms. Therefore, the identifications of *Cymbella minuta*, or *Encyonema minutum* or *Encyonema ventricosum* could be

considered correct. This proficiency test represents the first comparison of Italian operators about diatom analysis. This has underlined the difference between operators that have been working on diatoms least from twenty years and operators have been working on diatoms since the emanation of Legislative decree 152/2006; the firsts followed last updated taxonomy list; the others adopted those from Krammer *et al.* However this gap did not affect the results of ICMi values in term of consensus value. Other factor of variability in ICMi value was the uncertainty due to counting protocol adopted by participant: it is possible to count either valves or frustules as a diatom unit; diatoms to be counted should be around 400: total diatoms counted by each operator, vary from 331 to 507; other aspect is related to the treatment of broken diatoms, for example a broken valve or frustule can be excluded, counted only if of a frustule are present at least one pole and the central area. The results show that the main source of variability is due diatom taxonomy changes and counting protocols. The archiving of permanent slides and the creation of reference collections are particularly useful for the identification of difficult species as well as to make microphotography and create free iconographic databases [35]. The statistical analysis and the evaluation of z-score shown that most of the participants/operators results are comparable and this is very important, considering that such method had recently been introduced in the Regional Agencies. In order to assess operators' "performance", the z-score was calculated by using as reference value the assigned value for the total number of slides, namely the $ICMi = 0,24$ and the corresponding standard deviation = 0.01 (CV % = 4%). The results of z-score obtained in this PT can be explained as follows:

- 56% of participants obtained z-scores between -2 and 2, showing a good performance;
- 1% of participants obtained z-scores between |2| and |3|, and this result is considered as a "warning signal";
- 41% of participants obtained z-scores above 3 and below -3 have to consider a further training.

The data analysis of the case study gives the possibility to estimate the repeatability of each operator of the subset group. The repeatability value obtained is about 7% for each operator (excepted for operator n.2) and this is an acceptable result.

This evaluation was focused on taxonomic identification and counting of diatoms. Nevertheless, considering all the phases of the diatom index, including sampling, the variability of each operator become much higher. The use of reference materials and participation in proficiency tests are valuable tools to ensure reliability laboratories and comparability of analytical data products. Nationally currently there are no quantitative data on the use of reference materials and participation laboratories in PT and there are still few studies concerning quality assurance in the contest of biological monitoring of surface waters. As a consequence, this study highlights the need for the future to regularly organize both teaching opportunities and further meetings and learning events and demonstrates the importance of implementing the use of reference material collections to allow quality control and continuous progress in diatom identification.

CONFLICT OF INTEREST

The authors have no conflict of interest.

REFERENCES

1. CEC - Council of European Communities Directive 2000/60/EEC of 23 October 2000 establishing a framework for community action in the field of water policy. Luxembourg. Official Journal of European Communities; 2000.
2. Italy Legislative Decree No. 152 approving the Code on the Environment. Gazzetta Ufficiale della Repubblica Italiana No. 88, 14 April 2006.
3. Italy, Ministerial Decree (DM) 260/2010 of Nov. 2010. Regolamento recante « Criteri tecnici per la classificazione dello stato dei corpi idrici superficiali, per la modifica delle norme tecniche del decreto legislativo 3 aprile 2006, n. 152, recante norme in materia ambientale, predisposto ai sensi dell'articolo 75, comma 3, del medesimo decreto legislativo». Official journal n. 31, 7 February 2011.
4. Kelly MG, Juggins S, Guthrie R, Pritchard S, Jamieson J, Rippey B, Hirst H. and Yallop M. Assessment of ecological status in UK rivers using diatoms. Freshwater Biology 2008;53:403-422.
5. Van de Bund W. Water Framework Directive intercalibration technical report. European Commission Luxembourg: Office for Official Publications of the European Communities, 2009.
6. Kelly MG, Whitton BA. The Trophic Diatom Index: a new index for monitoring eutrophication in rivers. Journal of Applied Phycology, 1995;7: 433-444.
7. Dell'Uomo A, *L'Indice Diatomico di Eutrofizzazione/ Polluzione (EPI-D) nel monitoraggio delle acque correnti*. Roma. Linee Guida APAT, CTN AIM, 2004.
8. Rott E, Pfister P, van Dam H, Pipp E, Pall K, Binder N, Ortler K. *Indikationslisten für Aufwuchsalgen in Österreichischen Fließgewässern*, Teil 2: Trophieindikation und autökologische Anmerkungen Bundesministerium für Land- und Forstwirtschaft. Wien: Wasserwirtschaftskataster, 1999.
9. Kitner M , Poulíková A. Littoral diatoms as indicators for the eutrophication of shallow lakes. Hydrobiologia 2003;506-509: 519-524.
10. Blanco S, Ector L, Bécares E. Epiphytic diatoms as water quality indicators in spanish shallow lakes. Vie Milieu 2004;54:71-79.

11. DeNicola DM, Eyto E. Using epilithic algal communities to assess trophic status in Irish lakes. *Journal of Phycology* 2004;40: 481-495.
12. Prygel J, Coste M. Les diatomées et le diagnostic de la qualité des eaux courants continentales: les principales méthodes indiciaires. *Vie Milieu* , 1995;45:179-186.
13. Kauppila T, Moisio T, Salonen VP. A diatom-based inference model for autumn epilimnetic total phosphorus concentration and its application to a presently eutrophic boreal lake. *Journal of Paleolimnology* 2002;27: 261-273.
14. Besse-Lototskaya A P, Verdonschot F M, Sinkeldam J A. Uncertainty in diatom assessment: Sampling, identification and counting variation. *Hydrobiologia* 2006;566:247–260.
15. Mancini L, & Sollazzo C. Method for assessing the ecological state of current waters: diatomic communities. Istisan Report 09/19, Roma, 2009.
16. ISPRA. Biological methods for inland surface water. Guidelines Vol. 111/14, Roma, 2014.
17. EN 13946 . - Water quality. Guidance Standard for the routine sampling and pre-treatment of benthic diatom samples from rivers, European Committee for Standardization, Brussels. 2005
18. Krammer K, Lange-Bertalot H. Bacillariophyceae 1 Teil: Naviculaceae. In: Ettl H. (Ed.) *Süßwasserflora von Mitteleuropa* Stuttgart: Gustav Fischer-Verlag, 1986.
19. Krammer K, Lange-Bertalot H.. Bacillariophyceae 2 Teil: Bacillariaceae, Epithemiaceae, Surirellaceae. In: Ettl H. (Ed.) *Süßwasserflora von Mitteleuropa* Stuttgart: Gustav Fischer-Verlag, 1988.
20. Krammer K, Lange-Bertalot H. Bacillariophyceae 3 Teil: Centrales, Fragilariaceae, Eunotiaceae. In: Ettl H. (Ed.) *Süßwasserflora von Mitteleuropa* Stuttgart: Gustav Fischer-Verlag, 1991a.
21. Krammer K, Lange-Bertalot H.. Bacillariophyceae 4 Teil: Achnathaceae. Kritische Ergänzungen zu Navicula und Gomphonema. In: Ettl H. (Ed.) *Süßwasserflora von Mitteleuropa* Stuttgart: Gustav Fischer-Verlag, 1991b.
22. Krammer K, Lange-Bertalot H.. Bacillariophyceae 5 Teil: English and french translation of the keys. In: Ettl H. (Ed.) *Süßwasserflora von Mitteleuropa* Stuttgart: Gustav Fischer-Verlag, 2000.
23. Lange – Bertalot H. Ed. *Diatoms of Europe: diatoms of the European Inland Waters and Comparable Habitats* edited by Horst Lange – Bertalot Volume 1: Krammer, Kurt: The Genus *Pinnularia* Ruggell: Gantner Verlag, 2000.

24. Lange – Bertalot H. Ed..Diatoms of Europe: diatoms of the European Inland Waters and Comparable Habitats edited by Horst Lange – Bertalot. Volume 2: Lange Bertalot, Horst: Navicula sensu stricto, 10 Genera Separated from Navicula sensu stricto, Frustulia Ruggell: Gantner Verlag, 2001.
25. Lange – Bertalot H. Ed. Diatoms of Europe: diatoms of the European Inland Waters and Comparable Habitats edited by Horst Lange – Bertalot. Volume 3: Krammerr, Kurt: Cymbella Ruggell: Gantner Verlag, 2002.
26. Lange – Bertalot H. Ed. Diatoms of Europe: diatoms of the European Inland Waters and Comparable Habitats Elsewhere edited by Horst Lange – Bertalot. Volume 4: Krammerr, Kurt: Cymbopleura, Delicata, Navicymbula, Gomphocymbellopsis, Afrocybella, Supplements to Cymbelloid taxa Ruggell: Gantner Verlag, 2003.
27. EN 14407. Water quality. Guidance Standard for the identification, enumeration and interpretation of benthic diatom samples from running waters. European Committee for Standardization, Brussels, 2004.
28. CEMAGREF. Étude des méthodes biologiques d'appréciation quantitative de la qualité des eaux. Rapport Q. E. Lyon- A. F. Bassin Rhône- Méditerranée Corse. Lyon: CEMAGREF; 1982.
29. Kelly MG, Adams C, Graves AC, Jamieson J, J. Krokowski, Lycett E.B, Murray-Bligh J, Prithcard &Wilkins C. The trophic diatom index : a user's manual. Revised edition. Technical report E2/TR2. Environment Agency, Bristol, 2001.
30. Almeida S.F.P., Elias C., Ferreira J., Tornés E., Puccinelli C., Delmas F., Dörflinger G., Urbanič G., Marcheggiani S., Rosebery J., Mancini L., Sabater. S. Water quality assessment of rivers using diatom metrics across Mediterranean Europe: A methods intercalibration exercise. Science of The Total Environment 2014 Apr 1; 476-477:768-76.
31. Zelinka M, Marvan P. Zur Prazisierung der biologischen Klassifikation der Reinheit fliessender Gewasser. Arch. Hydrobiol. 1961;57: 398-407.
32. UNI EN ISO/IEC 17043. Conformity assessment - General requirements for proficiency testing. 2010.
33. Lindegarth M, Carstensen J, Johnson RK. Uncertainty of biological indicators for the WFD in Swedish water bodies: current procedures and a proposed framework for the future. Havsmiljöinstitutet, Sweden;2013. Deliverable 2.2-1, WATERS Report no. 2013:1

34. Kahlert M, Kelly M, Albert R-L, Almeida SFP, Bešta T, Blanco S, et al. Identification is a minor source of uncertainty in diatom-based ecological status assessments on a continent-wide scale: results of a European ring-test. *Hydrobiologia* 2012;695:109–24
35. Martone et al. Atlante delle diatomee bentoniche dei corsi d'acqua italiani. <http://www.isprambiente.gov.it/it/banche-dati/acque-interne-e-marino-costiere-1/atlante-delle-diatomee-bentoniche-dei-corsi-dacqua-italiani/index>.

SUPPLEMENTARY FILES

Table 4. List of diatom species identified

Code	Diatom species	Operator																															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	24	25	29	30	31	32					
ADMI	<i>Achnantheidium minutissimum</i> (Kützing) Czarnecki	13	25	18	47	70	36	32	32	68	51	33	37	36	19	31	12	44	42	22	16	30	20	18	44	36	53	15					
ADPY	<i>Achnantheidium pyrenaicum</i> (Hustedt) Kobayasi			14											7		24			8	15				6								
ADSA	<i>Achnantheidium saprophilum</i> (Kobayasi et Mayama) Round										1															4							
ADMS	<i>Adlafia minuscula</i> (Grunow) Lange-Bertalot																	3															
AINA	<i>Amphora inariensis</i> Krammer																		2	2	1												
AOVA	<i>Amphora ovalis</i> (Kützing) Kützing					1																											
APED	<i>Amphora pediculus</i> (Kützing) Grunow	1		4	1	2			3	1	2		3	3	4	4	1	2	2	1		4	4					1					
CPLA	<i>Cocconeis placentula</i> Ehrenberg									1										1		1											
CPLE	<i>Cocconeis placentula</i> Ehrenberg var <i>euyghypta</i>		1			1																			1								
CPLI	<i>Cocconeis placentula</i> var. <i>lineata</i> (Ehrenberg) van Heurck														4																		
CRAC	<i>Craticula accomoda</i> (Hustedt) Mann				2						2						1									5		1					
CAMB	<i>Craticula ambigua</i> (Ehrenberg) Mann		1																														
CHAL	<i>Craticula halophila</i> (Grunow e Van Heurck) Mann																		2	2	3												
CMLF	<i>Craticula molestiformis</i> (Hustedt) Lange-Bertalot				2																												
CATO	<i>Cyclotella atomus</i> Hustedt			2	3			2		1	4	13					13									4	4						
CYCL	<i>Cyclotella</i> F.T. Kützing ex. A. de Brébisson														17																		

[illegible]

[illegible]

FVUL	<i>Frustulia vulgaris</i> (Thwaites) De Toni																		2									
GACC	<i>Geissleria acceptata</i> (Hust.) Lange-Bertalot & Metzeltin										1																	
GDEC	<i>Geissleria decussis</i> (Ostrup) Lange-Bertalot & Metzeltin	1							1																			
GANG	<i>Gomphonema angustatum</i> (Kützing) Rabenhorst		1							2				1	2				24				1			3		2
GANT	<i>Gomphonema angustum</i> Agardh																			3	10		1					
GAUG	<i>Gomphonema augur</i> Ehrenberg		1																									
GMIC	<i>Gomphonema micropus</i> Kutzing var. <i>micropus</i>	2			2	3	1	2	2			1	2								2			3				
GMIN	<i>Gomphonema minutum</i> (Agardh) Agardh	2	4					x											5	5	15	1				3		
GOCU	<i>Gomphonema occultum</i> Reichardt & Lange-Bertalot	2	7																									
GOLI	<i>Gomphonema olivaceum</i> (Hornemann) Brébisson	15	12	8	16	13	16	20	26	23	12	14	13	27	25	14	26	6	42			30	13	30	18	9	20	22
GPAR	<i>Gomphonema parvulum</i> (Kützing) Kützing	4	4			1		2		3		5		3	3	6	2		2	7	1	3		2	1	1	18	1
GPRO	<i>Gomphonema productum</i> (Grunow) Lange-Bertalot & Reichardt																	1										
GPUM	<i>Gomphonema pumilum</i> (Gr) Reichardt Lange-Bertalot	1			3	1						5													2		2	
GROS	<i>Gomphonema rosenstockianum</i> Lange-Bertalot & Reichardt				3											6												
GTER	<i>Gomphonema tergestinum</i> Fricke		3			1	1	2		1	2	5		12			6			2			4		1	2		2
GTRU	<i>Gomphonema truncatum</i> Ehrenberg						1																		1			
HVEN	<i>Halamphora veneta</i> (Kützing) Levkov											4																
HCAP	<i>Hippodonta capitata</i> (Ehr.)Lange-Bert.Metzeltin & Witkowski						1																					
LMUT	<i>Luticola mutica</i> (Kutzing) D.G. Mann		1				1																					
LGOE	<i>Luticola goeppertiana</i> (Bleisch) Mann								1			1					2											

MAAT	<i>Mayamaea atomus</i> (Kützing) Lange-Bertalot				18	4	2		4		4	1			21											1	4	
MAPE	<i>Mayamaea atomus</i> var <i>permitis</i> (Hustedt) Lange-Bertalot	4		29		4	7				12						33	26		20								2
MVAR	<i>Melosira varians</i> Agardh					2																33	15	37				
MCIR	<i>Meridion circulare</i> (Greville) Agardh	3			1			1		1		1			4									1				
NANT	<i>Navicula antonii</i> Lange-Bertalot	1	1		1		2		2		1				9		3	1										10
NCPR	<i>Navicula capitatoradiata</i> Germain	1	2		1	1	1	2				1			2				4		1	1						1
NCIN	<i>Navicula cincta</i> (Ehrenberg) Ralfs																	2										
NCRY	<i>Navicula cryptocephala</i> Kützing												1		2													
NCTE	<i>Navicula cryptotenella</i> Lange-Bertalot	5	4	5	6	3		9	8	3	4	8	2	5	12	5	2	2	8	8	7	12	5		12	16	8	
NCTO	<i>Navicula cryptotenelloides</i> Lange-Bertalot													3	1							1						2
NERI	<i>Navicula erifuga</i>	1																										
NEXI	<i>Navicula exilis</i> Kützing																								1			
NGRE	<i>Navicula gregaria</i> Donkin	2	2	3	1	1	2	3	2	6			1	2	8		6		2			1		1				2
NLAN	<i>Navicula lanceolata</i> (Agardh) Ehrenberg							1																2				
NMEN	<i>Navicula menisculus</i> Schumann																		2		4							
NRAD	<i>Navicula radiosa</i> Kützing																			1								
NRCH	<i>Navicula reichardtiana</i> Lange-Bertalot	11	7	3	8	3	11	8	3	18	5	6	7	4	13	9	12	9	18	3		2		1	5			3
NTPT	<i>Navicula tripunctata</i> (Müller) Bory	1			2				1	1	1	3	1		5		2				2			1		8		1
NTRV	<i>Navicula trivialis</i> Lange-Bertalot															2												
NVEN	<i>Navicula veneta</i> Kützing						1	1					4										1		2			
NACI	<i>Nitzschia acicularis</i> (Kützing) W M Smith	17		1	2								4				1		16				1		1	2		13
NAMP	<i>Nitzschia amphibia</i> Grunow							1										8				1						
NIAN	<i>Nitzschia angustata</i> Grunow	1																										

NIAR	<i>Nitzschia archibaldii</i> Lange-Bertalot	3	23																									
NCPL	<i>Nitzschia capitellata</i> Hustedt	98		195	145	##	148	141	179	195	135	142	158	101	##	##	170		80	31	27			180	##	##	150	171
NCTN	<i>Nitzschia capitellata</i> var <i>tenuistrorsis</i>																						15					
NCOM	<i>Nitzschia communis</i> Rabenhorst					3		1																				
NCOT	<i>Nitzschia constricta</i> (Kützing) Ralfs	5	2	7						1																		
NDIS	<i>Nitzschia dissipata</i> (Kützing) Grunow	5	2	7	4	7	10	7	8	17	3	10	9	5	10	4	6	6	14	27	7	6	3	7	5	14	15	18
NFIL	<i>Nitzschia filiformis</i> (W M Smith) Van Heurck									1																		
NFON	<i>Nitzschia fonticola</i> Grunow	6		2	7	2	2	1	11		8	6	21	1	5		13	3		7							38	
NIFR	<i>Nitzschia frustulum</i> (Kützing)Grunow var. <i>frustulum</i>			1														6										
NHEU	<i>Nitzschia heufleriana</i> Grunow				1																							
NINC	<i>Nitzschia inconspicua</i> Grunow												1												3			
NLIN	<i>Nitzschia linearis</i> (Agardh) W Smith			1			1	1			1	3			4	1		3	4		1			6	7		1	
NPAL	<i>Nitzschia palea</i> (Kützing) W Smith	87	##	78	88	##	115	76	94	87	84	68	96	153	51	44	77	213	90	175	##	##		63	##	21	18	73
NPAD	<i>Nitzschia palea</i> (Kützing) W.Smith var. <i>debilis</i> (Kützing)Grunow														3							##						
NPAE	<i>Nitzschia paleacea</i> (Grunow) Grunow in van Heurck																	2					5					
NIPU	<i>Nitzschia pusilla</i> (Kützing) Grunow	1							3		1		1													2		
NREC	<i>Nitzschia recta</i> Hantzsch in Rabenhorst							1																	3			
NSOC	<i>Nitzschia sociabilis</i> Hustedt			1								7		13		2	6					5						
NSBL	<i>Nitzschia sublinearis</i> Hustedt																	4										
PTEL	<i>Planothidium ellipticum</i> (Cl.)Round & Bukhtiyarova													2														
PLFR	<i>Planothidium frequentissimum</i> (Lange-Bertalot)Round Bukhtiyarova	1	1									1	1					2										

PTLA	<i>Planothidium lanceolatum</i> (Kütz e Bréb) L-B		1	2						1		1			5				7	7	1	40	3	1				2
RSIN	<i>Reimeria sinuata</i> (Gregory) Kociolek Stoermer																2								1			
RUNI	<i>Reimeria uniseriata</i> Sala Guerrero & Ferrario										4																	
RABB	<i>Rhoicosphenia abbreviata</i> (Agardh) Lange-Bertalot									2										3								
SPUP	<i>Sellaphora pupula</i> (Kützing) Mereschkowsky	4	1		1		1			1	2	1			2		2								1		1	
SRPI	<i>Staurosira pinnata</i> Ehrenberg			1			2					1			1		1										2	
SANG	<i>Surirella angusta</i> Kützing	1	1	2			2	1	1	1		4	1		3	1			8	1	1		3		4	1	1	3
SBRE	<i>Surirella brebissonii</i> Krammer Lange-Bertalot	14	37	12	17	12	21	37	27	34			17	23	27	25		15		45	37	33		17	24	16	50	24
SBKU	<i>Surirella brebissonii var.kuetzingii</i> Krammer et Lange-Bertalot										18	22					16		28				20					
SLIN	<i>Surirella linearis</i> W M Smith									1																		
SUMI	<i>Surirella minuta</i> Brébisson				1																4				1	3		
SFAC	<i>Synedra fasciculata</i> (Kützing) Grunow									4																	3	
THAL	<i>Thalassiosira</i> P.T. Cleve									1																		
TPSN	<i>Thalassiosira pseudonana</i> Hasle Heimdal							26	1			19					49											
UBIC	<i>Ulnaria biceps</i> (Kützing) Compère									1																		