

darleq3: User Guide (Version 0.8.1)

Steve Juggins and Martyn Kelly

2017-09-14

1. Introduction

darleq3 is an R package for the assessment of river and lake ecological status using diatom data obtained by light microscopy (LM) or Next Generation Sequencing (NGS). The package contains functions to import diatom and associated environmental data from Excel worksheets, perform simple data validation checks, calculate various water quality metrics, EQRs and Water Framework Directive (WFD) quality classes for samples, and classification uncertainty for sites. The package can calculate Trophic Diatom Index TDI5LM, TDI4 and TDI3 scores for light microscopy river diatom samples, TDI5NGS for NGS river diatom samples, Lake Trophic Diatom Index LTDI2 and LTDI1 scores for light microscopy lake diatom samples, and Diatom Acidification Metric (DAM) scores for lake and river light microscopy samples. Details of the TDI / LTDI metrics, algorithm and derivation of the status class boundaries for rivers are given in Kelly *et al.* (2008) and for lakes in Bennion *et al.* (2014). Details of the DAM acidification metric is described in Juggins *et al.* (2016). Calculation of uncertainty of classification is described in Kelly *et al.* 2009.

darleq3 can be run in two ways, either as an interactive shiny app, or a series of R functions issues from the R console or an R script. The first method attempts to mimic the old DARLEQ2 software will be the easiest for most users. The second methods will be more convenient for processing multiple data sets, for automating darleq calculations, or including them in a longer chain of analysis.

2. Installation

The easiest way to install **darleq3** is from a github repository. To do this first install the package **devtools** with the following command, omitting the prompt (“>”):

```
> install.packages("devtools")
```

Then install **darleq3**. Note that this will also automatically install some additional packages on which **darleq3** depends.

```
> library(devtools)
> install_github("nsj3/darleq3", build_vignettes=TRUE)
```

darleq3 also contains an example Excel data file. This can be made available in a R session with the following:

```
> library(darleq3)
> fn <- system.file("extdata/DARLEQ2TestData.xlsx", package="darleq3")
```

The file can be opened in Excel using the following:

```
> # note running the following lines will open the file in Excel (if installed)
> shell.exec(fn)
```

3. Using the darleq3 Shiny app

darleq3 can run on a remote Shiny server or locally on a desktop PC running RStudio. The app will function in exactly the same way in both situations. To run **darleq3** on a remote shiny server open a web browser and point it to either:

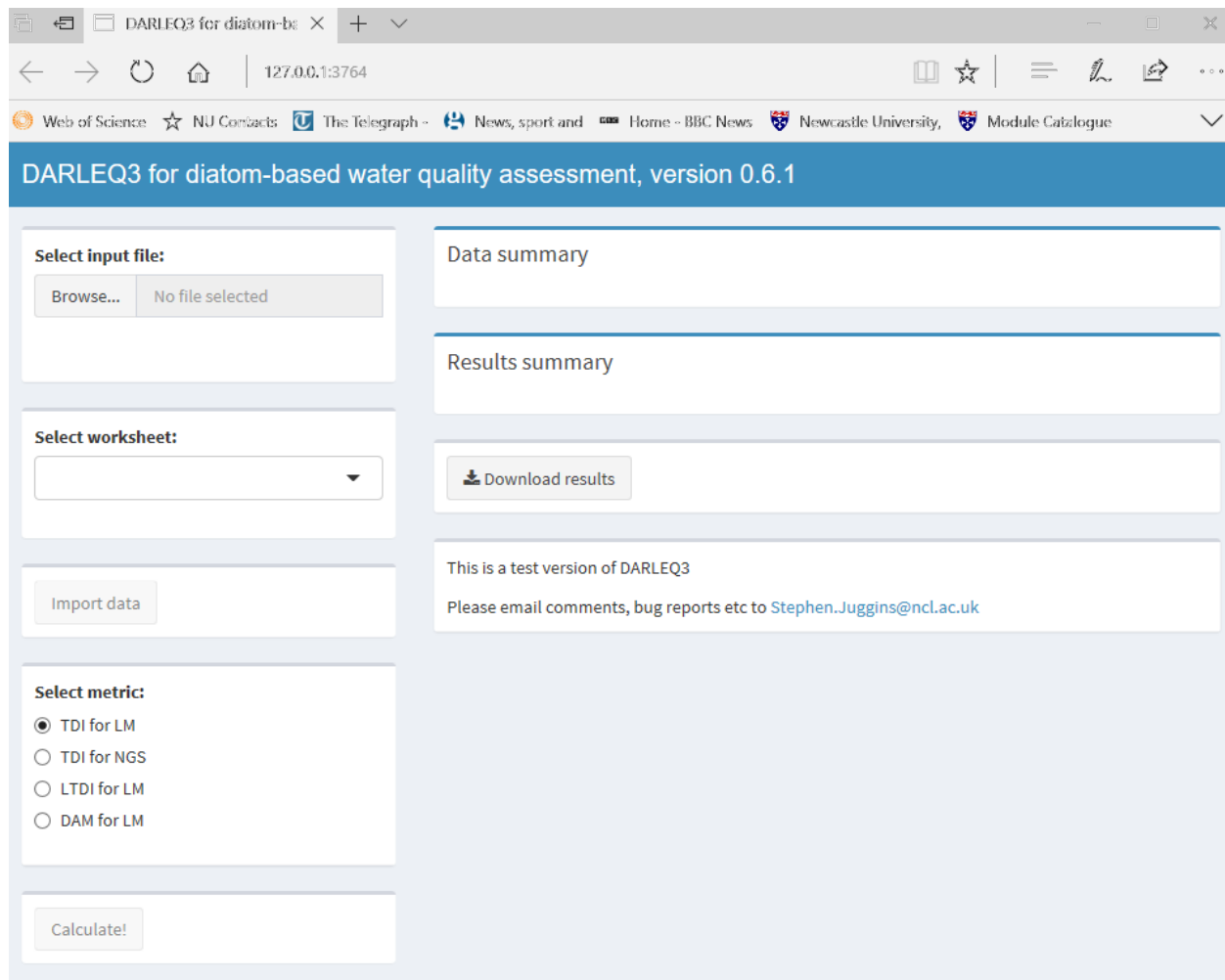


Figure 1: darleq3 shiny app

<https://nsj3.shinyapps.io/darleq3/>

<http://gpsgpuserver.ncl.ac.uk:3838/darleq3/>

Both these hosts have been set up for testing purposes and may change. There may be problems running the app on the second server listed from within the EA.

To run the app locally, simply start RStudio, load the `darleq3` package and run the command `runDARLEQ()`:

```
> library(darleq3)
> runDARLEQ()
```

This should open a browser and display the DARLEQ3 shiny app.

To use the app follow these simple steps:

- 1: Click the Browse... button to select and upload a DARLEQ diatom file (see below).
- 2: Once uploaded, select a sheet and click import. A summary (number of samples & taxa) will be displayed in the Data summary box when upload is complete.
- 3: Select the metric type. "TDI3 & 4 for LM" will calculate TDI3 and TDI4 for river samples according to the DARLEQ 2 taxon list, TDI5LM will calculate TDI5 for river LM diatom data, TDI for NGS will

calculate TDI5NGS for river NGS diatom data, “LTDI for LM” will calculate LTDI1 and LTDI2 for lake LM data, and “DAM for LM” will calculate the diatom acidification metric for river LM data. A summary of results will appear in the Results summary box when the calculations are complete.

- 4: Click Download Results to save the results in an Excel file. The default name for this file will be the “DARLEQ3_Results_” concatenated with the original data filename, worksheet name, and date.

To quit the app simply close the browser and or hit Escape in the RStudio Console window.

4. Using the darleq3 R package

darleq3 contains a number of functions for importing diatom data, calculating various sample and site-based metrics, EQRs and WFD quality classes, and saving the results in Excel format. The main functions are:

- **darleq** import diatom data from an Excel file, calculate metrics, EQRs and WFD quality classes, and save results in Excel format
- **read_DARLEQ** import diatom data from an Excel file
- **save_DARLEQ** save metric and EQR results in an Excel file
- **calc_Metric_EQR** calculate EQRs, WFD quality classes and summary diagnostic measures for multiple metrics
- **calc_Metric** calculate various diatom water quality metrics
- **calc_EQR** calculate sample and site EQRs and WFD quality classes
- **runDARLEQ** run DARLEQ3 as an interactive shiny app in a web browser

Type `?function_Name` at the R prompt to get help and example usage for these functions.

The **darleq3** functions have been designed to allow the user to perform individual steps of the data analysis sequence individually, for example importing diatom data, calculating a particular metric diatom from LM or NGS diatom data, or calculating EQRs from a metric and site information. These low-level functions are useful for embedding **darleq3** in a longer data analysis chain using R. The package also includes “wrapper” functions, that “wrap” multiple low-level functions to perform a complete analysis with a single function call.

4.1 darleq3 wrapper functions

The most useful wrapper function is **darleq**. This function imports data from an Excel file, calculates multiple metrics, EQRs and WFD classes and saves the results to another Excel file in one step.

```
> fn <- system.file("extdata/DARLEQ2TestData.xlsx", package="darleq3")
> darleq(fn)
```

darleq will, by default, import data from the first sheet in the Excel file, and calculate TDI3, TDI4 and TDI5LM. If the output filename is not given the function will generate a name by concatenating “DARLEQ3_Results_” with the original filename, the sheet name and the current date.

To specify the sheet name, a different metric, and an output file name:

```
> fn <- system.file("extdata/DARLEQ2TestData.xlsx", package="darleq3")
> darleq(fn, sheet="Lakes LTDI Test Data", metrics="LTDI2", outFile="Results.xlsx")
```

To calculate and save results for multiple metrics:

```
> fn <- system.file("extdata/DARLEQ2TestData.xlsx", package="darleq3")
> darleq(fn, sheet="Lakes LTDI Test Data", metrics=c("LTDI1", "LTDI2"), outFile="Results.xlsx")
```

4.2 darleq3 low-level functions

`darleq3` low-level functions are useful for calculating partial results or for embedding `darleq3` in a longer data analysis sequence. The key functions are `read_DARLEQ` to import data from a DARLEQ-formatted data file (see Section 6 below for guidelines on how to format the data correctly). `read_DARLEQ` returns a list with two elements: `diatom_data` - a data frame of the diatom count or relative abundance data, and `header` - a data frame of sample, site and environmental data from the header of the Excel file.

```
> fn <- system.file("extdata/DARLEQ2TestData.xlsx", package="darleq3")
> d <- read_DARLEQ(fn, "Rivers TDI Test Data")
> head(d$diatom_data[, 1:8])
```

```
##          AC023A AC083A AC143A AC161A AC9999 AD009A AM001A AM004A
## SPR001 0.000000      0      0      0      0 0.31348 0.000000 0.00000
## AUT001 0.000000      0      0      0      0 0.000000 0.000000 0.00000
## SPR002 0.332226      0      0      0      0 0.000000 0.332226 0.00000
## AUT002 0.000000      0      0      0      0 0.000000 0.000000 0.00000
## SPR003 0.000000      0      0      0      0 0.000000 0.000000 0.00000
## AUT003 0.317460      0      0      0      0 0.000000 0.000000 0.31746
```

```
> head(d$header)
```

```
##          SampleID SiteID SAMPLE_DATE Alkalinity Stream Reach
## SPR001 SPR001 36082 2010-04-14 242 KENNET Hambridge Rd., Newbury
## AUT001 AUT001 36082 2004-09-21 242 KENNET Hambridge Rd., Newbury
## SPR002 SPR002 34649 2004-04-02 408 LAMBOURN A4 Newbury
## AUT002 AUT002 34649 2004-09-21 408 LAMBOURN A4 Newbury
## SPR003 SPR003 36073 2004-04-02 213 LAMBOURN At Gauging Station, East Shefford
## AUT003 AUT003 36073 2004-09-21 213 LAMBOURN At Gauging Station, East Shefford
```

`calc_Metric_EQR` calculates one or more diatom metrics and the corresponding sample and site EQRS and WFD classes, and class uncertainties. The function returns a list with an element for each metric. Each element is itself a list containing sample EQRs, site EQRs and uncertainties and a job summary.

```
> fn <- system.file("extdata/DARLEQ2TestData.xlsx", package="darleq3")
> d <- read_DARLEQ(fn, "Rivers TDI Test Data")
> results <- calc_Metric_EQR(d, metrics=c("TDI4", "TDI5LM"))
> head(results$TDI5LM$EQR[, 9:15])
```

```
##          N_TDI5LM N2_TDI5LM Max_TDI5LM TDI5LM eTDI5LM EQR_TDI5LM Class_TDI5LM
## SPR001 41 12.39 16.93 55.60483 68.49447 1.0000000 High
## AUT001 23 3.09 51.57 70.01608 68.49447 0.7613627 Good
## SPR002 55 13.14 20.93 70.66666 69.28261 0.7639539 Good
## AUT002 39 7.69 26.91 66.08840 69.28261 0.8831896 High
## SPR003 39 9.37 27.30 49.94616 65.48689 1.0000000 High
## AUT003 32 5.87 37.14 39.65652 65.48689 1.0000000 High
```

```
> head(results$TDI5LM$Uncertainty)
```

```
##          SiteID N EQR WFDClass CoCH CoCG CoCM CoCP CoCB ROM CoCHG CoCMPB ROM_GM
## 43 36082 2 0.88 High 79.41 19.01 1.52 0.06 0.00 20.59 98.42 1.58 1.58
## 33 34649 2 0.82 High 58.61 38.23 3.09 0.07 0.00 41.39 96.84 3.16 3.16
## 42 36073 2 1.00 High 100.00 0.00 0.00 0.00 0.00 0.00 100.00 0.00 0.00
## 41 35965 2 1.00 High 100.00 0.00 0.00 0.00 0.00 0.00 100.00 0.00 0.00
## 36 35101 1 0.48 Moderate 0.06 14.04 62.37 23.35 0.19 85.96 14.10 85.90 14.10
## 35 35075 2 0.71 Good 14.23 71.48 14.05 0.23 0.00 28.52 85.72 14.28 14.28
```

`save_DARLEQ` saves the output from `calc_Metric_EQR` in an Excel file:

```
> fn <- system.file("extdata/DARLEQ2TestData.xlsx", package="darleq3")
> d <- read_DARLEQ(fn, "Rivers TDI Test Data")
> results <- calc_Metric_EQR(d, metrics=c("TDI4", "TDI5LM"))
> save_DARLEQ(results, outFile="Results.xlsx")
```

calc_Metric calculates a single metric from a data frame of diatom count or relative abundance data.

```
> fn <- system.file("extdata/DARLEQ2TestData.xlsx", package="darleq3")
> d <- read_DARLEQ(fn, "Rivers TDI Test Data")
> x <- calc_Metric(d$diatom_data, metric="TDI4")
> head(x$Metric)
```

```
##          TDI4
## SPR001 52.22581
## AUT001 83.44051
## SPR002 70.61036
## AUT002 67.53959
## SPR003 49.97693
## AUT003 38.45844
```

calc_EQR calculates sample and site EQRS and WFD classes, and class uncertainties from a list of sample metrics.

```
> fn <- system.file("extdata/DARLEQ2TestData.xlsx", package="darleq3")
> d <- read_DARLEQ(fn, "Rivers TDI Test Data")
> x <- calc_Metric(d$diatom_data, metric="TDI4")
> eqr <- calc_EQR(x, d$header)
> head(eqr$EQR[, 9:15])
```

```
##      N_TDI4 N2_TDI4 Max_TDI4      TDI4      eTDI4  EQR_TDI4 Class_TDI4
## SPR001   41   12.39   16.93 52.22581 68.49447 1.0000000      High
## AUT001   23    3.09   51.57 83.44051 68.49447 0.4204847  Moderate
## SPR002   54   13.05   20.93 70.61036 69.28261 0.7654202      Good
## AUT002   39    7.69   26.91 67.53959 69.28261 0.8453951      High
## SPR003   39    9.37   27.30 49.97693 65.48689 1.0000000      High
## AUT003   32    5.87   37.14 38.45844 65.48689 1.0000000      High
```

```
> head(eqr$Uncertainty)
```

```
##      SiteID N  EQR WFDClass   CoCH CoCG CoCM CoCP CoCB  ROM CoCHG CoCMPB ROM_GM
## 43  36082 2 0.71    Good  14.23 71.48 14.05 0.23 0.00 28.52 85.72 14.28 14.28
## 33  34649 2 0.81    High  54.37 42.03 3.54 0.07 0.00 45.63 96.39 3.61 3.61
## 42  36073 2 1.00    High 100.00 0.00 0.00 0.00 0.00 0.00 100.00 0.00 0.00
## 41  35965 2 1.00    High 100.00 0.00 0.00 0.00 0.00 0.00 100.00 0.00 0.00
## 36  35101 1 0.47  Moderate   0.04 11.90 61.95 25.89 0.23 88.10 11.93 88.07 11.93
## 35  35075 2 0.71    Good  14.23 71.48 14.05 0.23 0.00 28.52 85.72 14.28 14.28
```

5. Understanding darleq3 output

The DARLEQ shiny app and R functions produce output that is similar in structure and content to that produced by the DARLEQ2 program. Specifically, the shiny app and functions `darleq` and `save_DARLEQ` save data in an Excel file with the following content. For each metric, the output file will contain 3 worksheets, named `Code_Job_Summary`, and `Code_Uncertainty` (where `Code` is the code for each metric). These three sheets contain the following information:

5.1 Job summary

This sheet contains the input file name, worksheet name and a summary of the number of samples and taxa in the file. It also contains a list of taxa included in the file but excludes from the metric calculations either because they are planktic or because they are not included in the DARLEQ list of indicator values for that metric. The list also contains the number of occurrences (N), Hill's N2 effective number of occurrences (Hill 1973) and maximum abundance of these taxa. The list of useful in checking the data for coding errors to identify abundant taxa excluded from the metric calculations. For TDI3/4 and LTDI1/2 the output also contains a list of taxa with indicator values included in DARLEQ3 but not in DARLEQ2 software. This is useful in understanding the reasons for differences in metric scores between DARLEQ versions 2 and 3 for the same metric.

5.2 Sample_Summary

Sample Summary – this sheet contains metric, EQR and quality class results for each sample. First, the sample information listed in the original input file is repeated, and then results of the analysis are listed as follows (where CODE is the metric Code):

- Total_count: Sum of the counts or percentages of all taxa in a sample.

Percent_in_CODE: Percentages of the total count of taxa that are matched to taxa in the master taxon list and included in the metric calculations. If all taxa are matched this will be the same as the Total_count but will be less if, for example, planktic taxa are present. Comparison of these two fields will indicate if there are important taxa present in the sample but not included in the status calculations.

- N_CODE, N2_CODE, Max_CODE: Number of taxa (N), effective number of taxa (N2) and maximum abundance (max) of taxa included in the metric calculations.
- CODE: value of the metric for each sample.
- eCODE: Expected value of the metric for each sample according to typology (lakes) or site-specific prediction (rivers).
- EQR_CODE: EQR for each sample based on predicted and observed metrics.
- Class_CODE: Status class based on EQR.

After the metric and classification fields a series of summary fields are listed containing the percentage of various ecological groups of diatoms:

- Motile: Percentage of the motile diatoms in the sample.
- OrganicTolerant: Percentage of organic pollution tolerant diatoms in the sample.
- Planktic: Percentage of planktic diatoms in the sample. These are excluded from the status calculations.
- Saline: Percentage of diatoms tolerant of slightly saline waters.
- Comments: List of any warning messages generated during calculations for individual samples relating to missing or out-of-range environmental values.

5.3 Uncertainty

Multiple samples from each site are combined and an uncertainty analysis is performed using the mean EQR and number of samples according to Kelly *et al.* (2009):

- SiteID: Unique site code taken from row 2 of the input data.
- N: Number of samples for site used in calculation of mean EQR and CoC.

- EQR: Mean EQR for each site.
- lake_TYPE: lake type (only for lake data)
- WFDCClass: Status class based on mean EQR.
- CoCH - CoCB: Confidence that the site belongs to status class high, good, etc.
- RoM: Risk of misclassification for predicted class.
- CoCHG: Confidence that the site is better than moderate class.
- CoCMPB: Confidence that the site is moderate or worse class.
- RoM_GM: Risk of misclassification above / below the good / moderate boundary.

6. Input data format

`read_DARLEQ` and the shiny app import diatom data from an Excel file in either .xls or .xlsx format. An example Excel file is included in this package (see Section 2 on how to view it). The required data and layout are rather and are slightly different for river and lake samples. Figure 2 below shows the required format for performing TDI calculations for river samples.

The first four header rows are mandatory and must contain the following information:

- Row 1: SampleID: a short numerical or alphanumeric code to uniquely identify the sample. This field cannot be empty (an empty cell indicates the end of data).
- Row 2: SiteID: a short numerical or alphanumeric code to uniquely identify the site. This code will be used to aggregate multiple samples when calculating confidence of class for a site.
- Row 3: SampleDate: sample date in Day/Month/Year format. Missing dates are set to Spring for the purposes of classification using TDI3 and samples flagged with a warning.
- Row 4: Alkalinity: Mean annual alkalinity (or best available estimate) in mg l⁻¹ (CaCO₃). Missing values are set to 100 mg l⁻¹ for the purposes of classification and samples flagged with a warning. Alkalinity values outside the range of the site prediction algorithm are set to the appropriate limit (6 or 150 mg l⁻¹ for TDI3 and 5 or 250 mg l⁻¹ for TDI4 and TDI5LM / TDI5NGS).
- Rows 5+: Further option sample descriptors such as river name, reach name etc. These data are not used by the program but will be reproduced in the output.

Note that the second column of the header information must be left blank.

Identifiers for each row of the sample header information should be listed in column 1. Diatom data then follow the header information and may be in count or percentage format. The first column must contain the taxon code in either NBS or DiatCode (<http://www.ecrc.ucl.ac.uk/?q=databases/diatcode>) format. The codes in this column are used to link the data to the DARLEQ3 taxon list and ecological information and cannot be empty (an empty cell indicates the end of the data). The second column must include either the taxon name or code (ie. a repeat of column 1).

The remaining columns to the right of the taxon name contain diatom counts or percentages. Empty (blank) cells in the matrix will be read as zero. Character data in the diatom matrix will generate an error. A full list of diatom codes (either NBS or DiatCodes) are available in the data frame `darleq3_taxa`.

If the Diatom Acidification Metric (DAM) is to be calculated, the header must contain estimates of mean annual Calcium and DOC concentrations, rows named Calcium and DOC, and in ueq l⁻¹ and mg l⁻¹ respectively. Figure 3 shows an example formatted for calculation of TDI and DAM. Note that if only DAM scores are required the Alkalinity field may be left blank. Sample Date is not used for calculating DAM and may be left blank.

	A	B	C	D	E	F	G
1	SampleID		SPR001	AUT001	SPR002	AUT002	SPR003
2	SiteID		36082	36082	34649	34649	36073
3	SampleDate		14/04/2010	21/09/2004	02/04/2004	21/09/2004	02/04/2004
4	Alkalinity		242	242	408	408	213
5	Stream		KENNET	KENNET	LAMBOURN	LAMBOURN	LAMBOURN
6	Reach		Hambridge Rd	Hambridge Rd	A4 Newbury	A4 Newbury	At Gauging Sta
7	AC023A	Achnanthes conspicua var. conspicua	0	0	0.332226	0	0
8	AC083A	Achnanthes laevis	0	0	0	0	0
9	AC143A	Achnanthes oblongella	0	0	0	0	0
10	AC9999	Achnanthes sp.	0	0	0	0	0
11	AC161A	Achnanthes ventralis	0	0	0	0	0
12	ZZZ912	Achnanthidium biasolettiana	0	0	0	0	2.14724
13	AD009A	Achnanthidium microcephalum	0.31348	0	0	0	0
14	ZZZ835	Achnanthidium minutissimum type	16.9279	1.25786	7.30897	2.11082	27.3006
15	ZZZ911	Achnanthidium subatomus	0	0	0	0	0
16	AP001A	Amphipleura pellucida	0	0	0	0	0.306748
17	AM013A	Amphora inariensis	1.25392	0	1.3289	0	0
18	AM011A	Amphora libyca	1.5674	0	0.664452	0	0
19	AM084A	Amphora montana	0	0	0	0	0
20	AM001A	Amphora ovalis var. ovalis	0	0	0.332226	0	0
21	AM012A	Amphora pediculus	3.76176	0	5.31561	3.16623	3.37423
22	AM9999	Amphora sp.	0	0	0	0.263852	0
23	AM004A	Amphora veneta var. veneta	0	0	0	0	0

Figure 2: Example format for river diatom samples

	A	B	C	D	E	F	G
1	SampleID		UK002_90	UK002_91	UK002_92	UK003_90	UK003_91
2	Site		UK002	UK002	UK002	UK003	UK003
3	Date		1990	1991	1992	1990	1991
4	Alkalinity						
5	Calcium		43.66	40.67	44.06	55.89	58.38
6	DOC		2.75	1.66	2.4	3.4	3.6
7	AC083A	Achnanthes laevis	0.0	0.0	0.6	0.0	1.3
8	AC143A	Achnanthes oblongella	0.6	1.0	1.2	49.5	56.4
9	AC148A	Achnanthes modestiformis	0.0	1.0	1.2	0.0	0.0
10	AC9999	Achnanthes sp.	0.0	0.3	0.0	0.3	0.0
11	BR001A	Brachysira vitrea	0.0	0.0	0.0	3.7	0.0
12	BR006A	Brachysira brebissonii fo. brebissonii	0.0	0.0	0.0	0.0	0.0
13	CM004A	Cymbella microcephala fo. microcephala	0.0	0.0	0.0	0.0	0.0
14	CM009A	Cymbella naviculiformis	0.0	0.0	0.0	0.0	0.0
15	CM014A	Cymbella aequalis	0.0	0.0	0.0	0.0	0.0
16	CM9999	Cymbella sp.	0.0	0.0	0.0	0.0	0.0

Figure 3: Example format for river diatom TDI and DAM samples

	A	B	C	D	E	F	
1	SampleId		ACHNAU4R	ACHNSP4P	ACHNSP4R	ACHNSU4R	AILS
2	SiteId		14403	14403	14403	14403	
3	SampleDate		08/11/2004	15/04/2004	15/04/2004	07/09/2004	04/1
4	Type		MA	MA	MA	MA	MA
5	AC001A	Achnanthes lanceolata	0.26178	0	0.455063	0	
6	AC006A	Achnanthes clevei	0.26178	0.552486	0	0	
7	AC007A	Achnanthes oestrupii	0.52356	0	0	0	
8	AC013A	Achnanthes minutissima	42.1466	26.2431	45.2787	56.0606	1
9	AC016A	Achnanthes delicatula	0	0	0	0	
10	AC022A	Achnanthes marginulata	0	0	0	0	0.
11	AC023A	Achnanthes conspicua	0	0	0	0	
12	AC025A	Achnanthes flexella	0	0	0	0	0.
13	AC034A	Achnanthes suchlandtii	0	0	0	0	
14	AC035A	Achnanthes pusilla	0	0.828729	0.227531	0	

Figure 4: Example format for lake diatom LTDI samples

The required input format for lake samples is shown in Figure 4. This is exactly the same as for river data except that the fourth row must be named LAKE_TYPE and contain a code indicating lake type according to the GB lake typology alkalinity classes. Marl lakes are included in the high alkalinity (HA) group. Peat and brackish lakes are not covered by the tool. Sample date for lake samples is not used in the class calculations and can contain missing values.

7. Acknowledgements

8. References

- Bennion, H., Kelly, M.G., Juggins, S., Yallop, M.L., Burgess, A., Jamieson, J., Krokowski, J., 2014. Assessment of ecological status in UK lakes using benthic diatoms. *Freshwater Science* **33**, 639-654.
- Juggins, S., Kelly, M., Allott, T., Kelly-Quinn, M., Monteith, D., 2016. A Water Framework Directive-compatible metric for assessing acidification in UK and Irish rivers using diatoms. *Science of The Total Environment* **568**, 671-678.
- Kelly, M., Bennion, H., Burgess, A., Ellis, J., Juggins, S., Guthrie, R., Jamieson, J., Adriaenssens, V., Yallop, M., 2009. Uncertainty in ecological status assessments of lakes and rivers using diatoms. *Hydrobiologia* **633**, 5-15.
- Kelly, M., Juggins, S., Guthrie, R., Pritchard, S., Jamieson, J., Rippey, B., Hirst, H., Yallop, M., 2008. Assessment of ecological status in UK rivers using diatoms. *Freshwater Biology* **53**, 403-422.