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Italian reference rivers under the Water Framework Directive umbrella: do natural factors actually depict the observed nutrient conditions?

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Abstract

Background: Despite the efforts made in the last century to counteract the nutrient enrichment from diffuse and point-sources, the excess of nitrogen and phosphorous is among the main causes of degradation of European rivers. In this context, determining natural background concentrations of nutrients in rivers is crucial for a correct definition of their ecological status. In the most anthropized regions, this is a difficult task. This study provides a nation-wide assessment of the nutrient concentration variability between Italian river reference sites.

Results: We applied the Affinity Propagation technique to identify groups of river sites classified as reference based on measured nutrients and oxygen water saturation. The role of natural and anthropogenic factors determining differences in nutrients concentration between groups of sites was explored. Nitrate concentrations varied from 0.01 mg N l^{-1} to more than 5 mg N l^{-1}. Ammonia and total phosphorous varied between 0.001 and 0.12 mg l^{-1}. Observed nutrient levels, although in line with those identified for reference sites in other countries, largely exceed the ranges reported for natural basins. Atmospheric deposition of inorganic N and artificial and/or high-impact agricultural land use are the major factors determining differences in nutrient concentration. Factors like, e.g. catchment size, precipitation amount and altitude do not play a relevant role in explaining nutrient differences between groups of reference sites.

Conclusions: We especially focused on (i) major causes of failure in the selection of appropriate reference sites in Italy; (ii) the potential of setting higher NO_3-N thresholds for the classification of ecological status in specific areas, and (iii) the prospective of a regionalization approach, in which human effects are accepted to a low degree for reference site selection or when setting thresholds for peculiar geographical areas.

Keywords: WFD, Ecological status, Land use, Atmospheric deposition, Nitrate

Background

Despite more than 20 years of European policies contrasting water quality deterioration (e.g. Nitrates Directive, 91/676/EEC; Urban Waste Water Treatment Directive, 91/271/EEC; Water Framework Directive, 2000/60/EC) nutrient enrichment from diffuse- and point-sources remains one of the main reasons for the degradation of European water bodies, including rivers [1]. Nutrient enrichment interferes with the achievement of environmental goals by directly affecting biological communities eventually determining eutrophication problems. The Water Framework Directive (WFD) specifically addresses this issue, legally requiring European member states to prevent further deterioration and

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restore water bodies not meeting environmental objectives. These objectives include maintaining nutrients levels which do not preclude the achievement of good status as defined by biological communities. However, the WFD does not provide a common approach to monitor nutrients in relation to ecological status.

The WFD postulates that the assessment of ecological status must be based on the comparison between reference and observed conditions, a well-established approach worldwide [2, 3]. Reference conditions should represent nearly natural and undisturbed environments, not or minimally affected by anthropogenic disturbance. These conditions are subject to ample natural variability mainly depending on the geographical context and climate conditions [2, 4]. Such variability is often clearly mirrored by biological elements [5] and it is also somewhat expected to affect nutrient levels [6]. Several basin characteristics (e.g. geology, vegetation, climate) influence the processes, both biotic and physical, that control nutrient concentrations in pristine rivers [7–10]. However, nitrogen and phosphorus are often limiting elements, scarcely available in natural environments because subject to consumption by biological communities. Therefore, in unaltered waterbodies, the expected variability of nutrients concentration is very low.

In accordance to the WFD, a type- (or site-) specific approach should be considered for biological elements. The scope of defining a typology for water bodies is to provide coherent and comparable geographical units able to represent the broad variety of ecological conditions observed in Europe. The use of types is partly to limit within-type natural variation to make statistical comparison more effective. The typological approach is therefore expected to support a better understanding of the response to alterations, especially for biological communities. Within this framework, the accuracy in assessing the ecological status is linked to the availability of reference sites and to a suitable description of reference conditions across types. The current European context to assess ecological conditions reveals a wide variability of nutrients thresholds between countries and usually those thresholds are not specifically related to river types [11]. A special effort was dedicated to verify compliance of biological methods to the WFD, while the conditions representing good ecological status for nutrients have been far less studied. Moreover, it is widely recognized that finding pristine sites to set reference conditions can be difficult or impossible [12, 13]. In the United States, for example, excess of nutrient exports, coupled with widespread atmospheric N deposition, often preclude the chances to identify pristine reference streams and watersheds [14–16]. Accordingly, reference sites are often selected based on the 'best available' or 'least disturbed' condition [12, 17], and agreed thresholds of anthropogenic influence indicators are set to accept sites as reference for biological communities [18–20].

Another important principle guiding the WFD is that appropriate management measures should be put into practice to restore water bodies or prevent their further degradation, thus there is a need for reliable tools to measure the distance between reference and observed conditions. This is a key point to understand the efficacy of the applied restoration measures. In this context, setting benchmark values for nutrient parameters that affect the status of water quality is crucial [11].

In Italy a high proportion of rivers is considered compromised, with 57% of the rivers monitored in the last decade not reaching the "good" ecological status [21]. Considering this context, the present paper provides elements specifically related to evaluating near-natural conditions for nutrients in Italian rivers, to support the assessment of ecological status. The study includes data from Italian reference sites along an extensive North–South geographical gradient and covers a wide gamut of river types sensu WFD. We specifically aimed to: (1) explore the variability of nutrient concentrations under reference conditions, and (2) identify the main natural and anthropogenic factors influencing nutrient concentrations observed in reference sites. Additionally, we indirectly assessed differences in nutrients concentration between Italian river macro-types.

Materials and methods

Study sites

Study sites cover a wide portion of the Italian territory from north to south, plus one of the two major Italian islands, Sardinia (Fig. 1). Site coordinates are reported in Additional files (Additional file 1: Table S1). Sites represent a comprehensive variety of geographical features and range from Alpine streams to Mediterranean temporary rivers. All sites were classified as 'Reference sites' by competent Regional and National authorities. Designation of reference stream sites in Italy follows a set of international standards [19, 20, 23] and national guidelines [24, 25]. Reference sites are identified as river reaches showing non-significant anthropic disturbance based on the verification of a set of quantitative and qualitative criteria on which two thresholds ('reference' and 'rejection' threshold) are established [24]. Criteria are grouped in alteration categories that include point and diffuse pollution, riparian buffer strip condition, hydromorphological alterations, water abstraction, discharge regulation, biological pressures and other alterations. Sites were also classified in river types according to the National Italian Decree 131/2008 (DM 131/2008). Types can be grouped in broader categories according

Fig. 1 Sampling sites location. Colors identify river macro-types, broadly defined according to the WFD Intercalibration categories. Please refer to Table 1 for group coding

to macro-types defined by the EU WFD intercalibration process [22] and formally recognized in Italy within the Decree 260/2010 (DM 260/2010). Eight river categories, covering all Italian macro-types with the exclusion of medium-sized lowland Mediterranean rivers and grouping more than 60 different National river types, were considered (Table 1). Groups include 6 formally identified Italian macro-types and 2 extra types based on specific features, namely Alpine streams with significant glacier influence (i.e., A2_GH) and lowland streams not fed by springs (i.e., C_mix). This selection guarantees consistency and a sufficient number of sites within each group for analysis. The legally binding typological classification for the investigated sites was performed by authorities who validated and provided the data.

The reference sites dataset was assembled from two sources. The primary source was the Italian Informative System for Water Protection [26]. SINTAI is a national repository containing data on water bodies collected by Italian Environment Agencies. A set of reference sites data, relevant for this study, was directly provided by the Italian Institute for Environmental Protection and Research, ISPRA. Additional data were obtained from CNR-IRSA research projects database. The final dataset incorporated data from 154 locations (sites) on 138 rivers. We used water quality data from more than 1000 samples collected between 2008 and 2016. Most of the sites were sampled at least seasonally for more than one year, 6% of sites in M5 macro-type were sampled in one occasion only. Where multiple samples were collected

Table 1 Description of Italian river macro-types considered in this paper (modified from EC, 2018 and DM 260/2010)

Type	River characterization	Distance to source	Catchment area	Catchment altitude and substrate type	Alkalinity/geology	Flow regime	# Sites
A1	Pre-Alpine, small- to medium- sized, calcareous	< 75 km	10–1000 km^2	800–2500 m; boulders/cobble	High (but not extremely high) alkalinity		15
A2	Alpine, small- to medium-sized, high altitude, siliceous	< 75 km	10–1000 km^2	max 3000 m, mean 1500 m; boulders	Non-calcareous (granite, metamorphic) medium to low alkalinity	Nival--glacial	28
A2_GH	Alpine, small-sized, high altitude, siliceous, with glaciers influence	< 25 km	< 150 km^2	max 3000 m, mean 1700 m; boulders	Non-calcareous (granite, metamorphic) medium to low alkalinity	Nival–glacial	9
C1	Very small siliceous lowland rivers in the Po plain (North of the Po River)	< 15 km	< 15 km^2	Mean ≈ 150 m; sandy to gravel	Siliceous	Spring-fed origin	11
C_mix	Other lowland rivers (excluding C1 and non-wadeable rivers) in the Po plain (North of the Po River)	Any	Any	Mean ≈ 200 m; sandy to gravel/ rock	Mixed	Not spring-fed	9
M1	Small-sized Mediterranean streams	< 25 km	≈ < 160 km^2	Any	Mixed (except siliceous)	Highly seasonal	43
M4	Medium-sized Mediterranean mountain (no lowlands) streams	25–75 km	≈ 160 – 760 km^2	Mean ≈ 400 m/Any	Non-siliceous	Highly seasonal	12
M5	Temporary streams (Mediterranean region)	Any	Any	Any	Any	Temporary	27

from a single site, i.e., in different seasons and years, values of single parameters derived from different seasons and years were averaged.

Variables included in the analyses

To define the physicochemical variation among river sites, we considered the following variables: O_2 saturation, ammonia (NH_4-N), nitrate (NO_3-N) and total phosphorous (TP). Such variables, whose assessment is mandatory in Italy for WFD purposes, are usually available for all Italian monitoring sites. The LIMeco descriptor [27] was also considered in the analyses. It is calculated by assigning a score to the concentration of the four above-mentioned physicochemical variables. Scores are then averaged to obtain the final LIMeco descriptor, ranging from 1 (high quality) to 0 (bad quality) (Additional file 1: Table S2). Thresholds to assign the score to the concentration of single physicochemical variables are set at the 75th percentile (or 90th for TP) of concentration values observed in a pool of reference samples (≈ 100) collected for research purposes before 2008 [24] and not considering a differentiation between river types.

For the interpretation of the physicochemical variation among river sites, climatic data, land-use information and nitrogen atmospheric deposition fluxes were considered. Data for rainfall and temperature were obtained from the JRC's Catchment Characterisation and Modelling River and Catchment database for Europe—CCM2 [28] that derives layers from digital elevation data and ancillary information. Climatic variables are based on the WORLDCLIM database [29] providing interpolated climate surfaces for global land areas referring to the 1950–2000 period. Multi-polygon shapefile containing bands with climatic data were downloaded and values for investigated sites—as positions in a vector point layer—extracted. Elevation for most of the investigated sites were available from the data providers. Missing data for elevation and slope values for all sites were calculated from a 20 m Digital Elevation Model.

Land use data were obtained from a 3rd level CORINE Land Cover inventory map (2012 update) of the Italian territory. Shapefiles of the sites' watershed were available or newly delineated and catchment areas calculated. Percentages for each 3rd level land cover class were extracted for each catchment intersecting CORINE shapefile with the basin shapefile. Percentages were summed up into four categories: natural land uses (Corine category 3, 4 and 5); agriculture with low impact (Corine category 2.3 and 2.4); agriculture with high impact (Corine categories 2.1 and 2.2) and artificial land uses (Corine category

1). An index of land use modification in the catchment (Land Use Index catchment; LUIc) was calculated for all sites. The index follows the scoring system described in [30] and [31] and assigns a score to non-natural land uses. LUIc values range from 0 (100% natural) to 5 (100% urban).

Fluxes of reduced (NH_4-N) and oxidized nitrogen (NO_3-N) in the wet deposition at the investigated sites were derived from EMEP MSC-W chemical transport model [32]. Map of annual wet deposition for year 2017 was downloaded as raster file and values of nitrogen extracted for investigated sites as positions in a vector point layer. Data referred to year 2017 were considered as an average approximation of the wet deposition in the research period. We used the sum of NH_4-N and NO_3-N to obtain the flux of the inorganic nitrogen (N_{in}).

All geometry, geoprocessing and sampling analysis were performed in QGIS environment (QGIS.org, 2018). The set of environmental variables considered for analysis is reported in Table 2.

Data analysis

Multivariate classification and ordination of river sites

To highlight possible discontinuities and to indirectly match existing, in-use typologies, we adopted a classification approach to identify groups of river sites based on nutrient and oxygen water concentration. Concurrently with the group definition, we were interested in identifying representative example sites able to illustrate actual river conditions. Nutrient concentrations and oxygen saturation were used to perform Affinity Propagation (AP), exemplar-based agglomerative clustering [33], to define groups. AP was based on similarity between data samples, measured with negative Euclidean distance (squared error). Clustering was implemented using the 'apcluster' package [34]; this and further analysis were performed in the R v.3.6.3 environment (R Core Team, 2019). Affinity propagation is an iterative method that aims at maximizing the net similarity. For each obtained cluster, the exemplar sample is chosen among the observed data samples and not computed as hypothetical average [35]. To aid interpretation of results, we aimed at selecting a reasonably low number of clusters. Thus, the solution with the smallest number of clusters providing the highest net similarity was selected.

Prior to analysis, physicochemical and environmental variables were log-transformed, apart from percentages, for which a logit transformation was used ($\log(y/[1-y])$) [36]. To make descriptors comparable, variables were then standardized to z-scores [37].

Based on the same data and measure of similarity used for AP clustering, we explored the location of groups of sites with Principal Coordinate Analysis (PCoA; 'cmdscale' in the 'stats' package v. 3.6.2, R Core Team, 2019). This is a multivariate technique that can represent the relationships among sites and supports the projection of variables on the spatial ordination of the sites (or groups of sites). A posteriori, the studied variables were thus related to the PCoA axes using a permutation-based vector fitting technique ('envfit', in vegan R library), and those showing a significant association to the axes were drawn on the ordination plot. This strengthened the

Table 2 Environmental variables included in the analyses

Category	Variable [unit]	Code	Notes
Water physicochemical	Oxygen saturation [%O_2]	DO	Analytical methods [80]
	Ammonia [mg l^{-1} NH_4-N]	NNH4	
	Nitrate [mg l^{-1} NO_3-N]	NNO3	
	Total phosphorous [mg l^{-1} P-tot]	TP	
	LIMeco (score)	LIMeco	[27] (Additional file 1: Table S2)
Climatic	Rain [mm]	Rain	Max, mean, min and standard dev (sd) 1950–2000 period—CCM2 database [28]
	Air temperature [°C]	Temp	
Geographical	Catchment area [km^2]	Catch	From data provider or derived form QGIS
	Site altitude [m a.s.l.]	Alt	
	Slope of the river [%]	River_Slope	
Land use catchment	Land use artificial [%]	LU_art	[30, 31]
	Land use agriculture low impact [%]	LU_agri_hi	
	Land use agriculture high impact [%]	LU_agri_low	
	Land use natural [%]	LU_nat	
	Land Use index [score]	LUIc	
Atmospheric deposition	N_{in} atmospheric deposition [mgN $m^{-2}y^{-1}$]	WetDEP_N	EMEP database [32]

interpretation of which environmental factors are more relevant in explaining nutrient variation between river sites.

Comparing variables between AP clusters

The BDM test [38] was used to test differences in the studied environmental variables between AP clusters (R package 'asbio', [39]). This nonparametric test on ranks extends the classical ANOVA approach to heteroscedastic designs with unequal cell frequencies. Post hoc comparison was implemented with Dunn [40] and Benjamini and Hochberg, [41] correction for false discovery rate (FDR; R package 'dunntest', [42]).

Results

Nutrient and oxygen levels at reference sites

Table 3 presents some statistical descriptors of nutrient data from the 154 reference sites. NO_3-N concentrations varied of three orders of magnitude, from values close to the detection limit (i.e., few micrograms) to more than 5 mg l^{-1}. Nevertheless, 50% of sites showed concentrations falling in a narrow range (0.2—0.9 mg l^{-1}). NH_4-N is one order of magnitude lower than NO_3-N and its proportion in inorganic nitrogen (N_{in}) is about 4% (median values). NH_4-N and TP exhibited the same range of variation (DL—0.12 mg l^{-1}) and mean (0.03 mg l^{-1}) and they were significantly correlated (Pearson's $r=0.27$, $p=0.0006$). Variability of oxygen saturation was lower than that of nutrients, with 90% of river sites showing values higher than 85%. A significant negative correlation between nitrate and oxygen saturation was found (Pearson's $r=-0.28$, $p=0.0003$).

As expected, all four analyzed parameters were well related to the LIMeco index, although the relation between NO_3-N and LIMeco presented the highest significance (Pearson's $r=0.67$, $p<0.0001$).

Table 3 Main statistical descriptors of nutrients and oxygen saturation (DO) data at the 154 reference sites

	DO %	NH_4-N mg l^{-1}	NO_3-N mg l^{-1}	TP mg l^{-1}
Mean	96.09	0.026	0.666	0.030
SD	9.89	0.021	0.784	0.024
cv%	10.3	80.6	117.7	77.6
Minimum	65.0	0.001	0.005	0.001
10^{th}	85.25	0.007	0.140	0.006
Median	97.58	0.020	0.403	0.028
75^{th}	100.0	0.030	0.879	0.049
90^{th}	104.9	0.047	1.392	0.060
Maximum	146.00	0.126	5.640	0.128

Nutrient-based reference sites clustering and association to environmental and anthropogenic factors

Graphical representations of the results of Affinity Propagation (AP) clusters and principal coordinate analysis (PCoA) ordination on nutrient concentrations and oxygen saturation are shown in Figs. 2 and 3. The comparatively smaller number of groups providing the higher net similarity led to the 8 clusters solution (Additional file 1: Material S3). Such groups are displayed in Fig. 2 with the group label approximately located on the cluster exemplar site that is connected (lines) to other cluster members. Each cluster is characterized by a different color. Colors reflect group attribution to a water quality class (blue: high; green: good; yellow: moderate), based on the LIMeco descriptor (please see Sect. 2.2. and Additional file 1: Table S2). The positioning of the groups with respect to vectors representing nutrient concentrations and oxygen saturation within the multivariate space identified by the first two PCoA axes is shown. Groups 6–8 were discriminated from others by higher NO_3-N concentration and lower oxygen saturation levels. Group 3 had higher oxygen saturation levels than groups 1, 2 and 4. A comparatively higher concentration of NH_4-N and TP characterized group 5.

Among the studied environmental and anthropogenic variables, some were significantly associated to the PCoA axes (Additional file 1: Table S4) and are shown as vectors in Fig. 3 together with the AP groups (polygons, with same colors of spider-plots in Fig. 2). Variable significance and proximity to groups in the ordination diagram support the interpretation of major factors behind variation of nutrients and groups meaning. Groups 6 to 8 align with the vector of nitrogen atmospheric deposition, that is highly correlated with NO_3-N concentration (Pearson's $r=0.60$; $p<0.001$). Group 5 is associated with the presence of artificial land uses in the catchment. More in general, increased anthropogenic pressures (left side of the diagram) differentiate groups 5 to 8 from the others. Differences in location on the diagram of the remaining groups (i.e., clusters 1–4) seem related to the variation of natural factors (e.g., altitude, river slope) and to the proportion of natural land uses in the catchment. The LIMeco descriptor vector, that is clearly aligned to PCoA axis 1, suggests a water quality gradient between river sites and AP groups, with quality gradually increasing from left to right.

The BDM test, run on all environmental variables of Table 2, emphasized significant differences in AP groups for land uses, nitrogen deposition, water quality (i.e., LIMeco) and typological features. Significant differences between groups for climatic variables were found only analyzing the standard deviation of such variables. The variability of selected features in the different AP groups

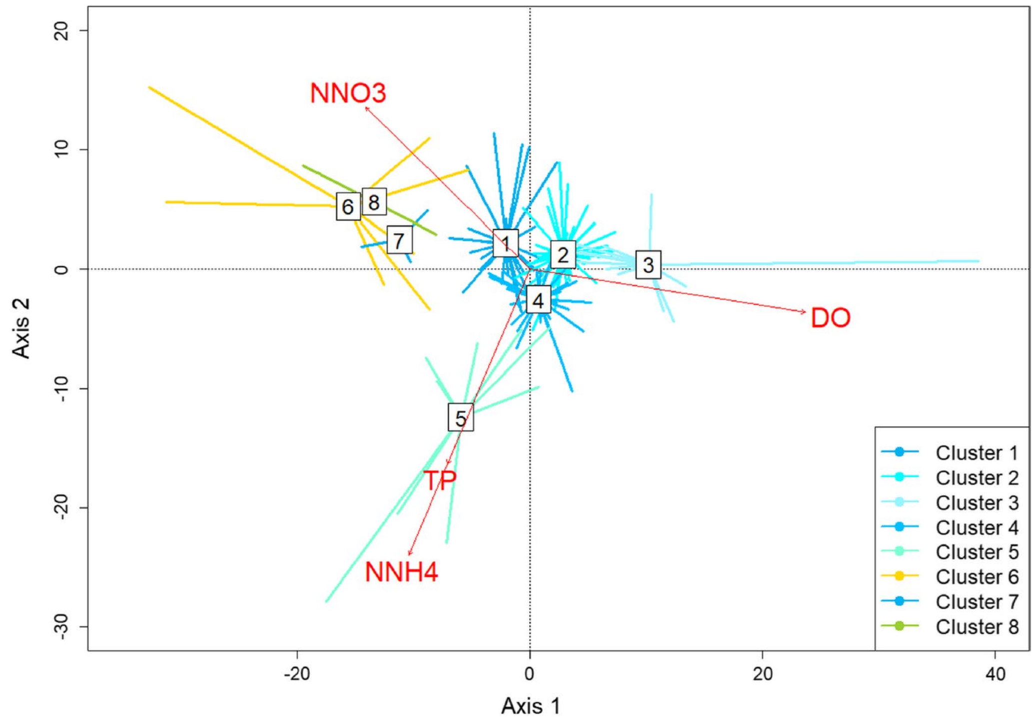

Fig. 2 Affinity propagation (AP) clusters positioning in PCoA space as determined by nutrients concentration and oxygen saturation (see Table 2 for codes meaning)

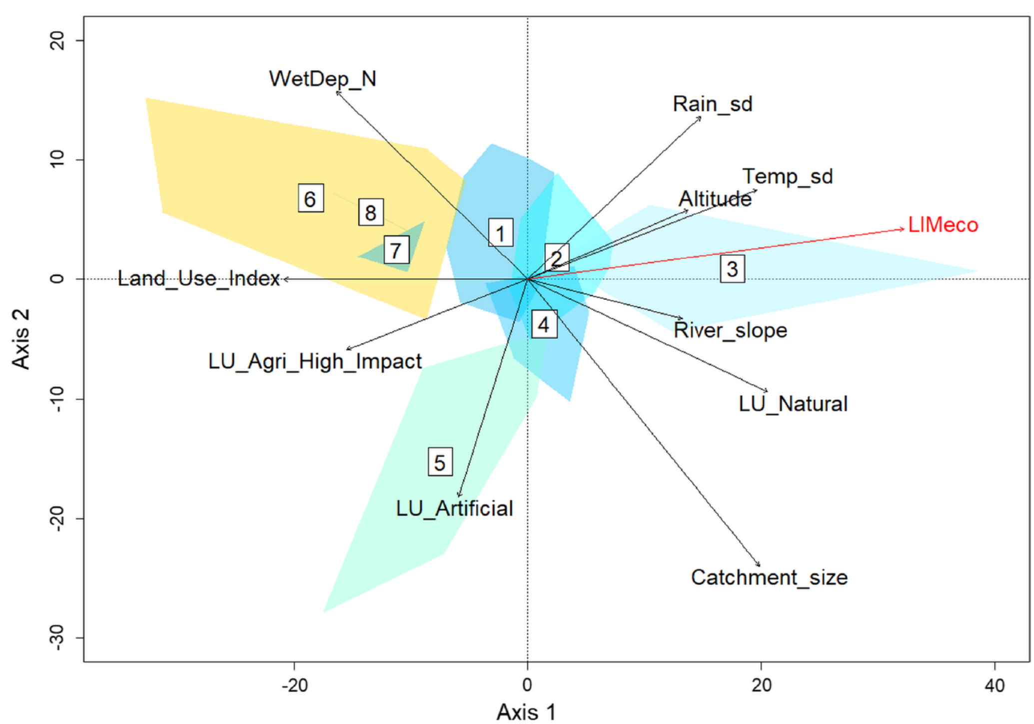

Fig. 3 Affinity propagation (AP) clusters positioning in PCoA space. Arrows represent variables significantly related to PCoA axes. LIMeco is reported in red since it is derived from the physicochemical variables used for clustering and PCoA ordination

is displayed in Figs. 4 and 5, including an indication of between-group differences (Dunn's test results). BDM and Dunn's test results on differences between AP groups for the whole set of environmental variables are reported as Additional file S5.

The variability in selected typological and climatic features is represented in Fig. 4 jointly with water quality expressed by LIMeco. AP groups were ordered by increasing water quality (i.e., LIMeco) from left to right. Statistically significant differences between AP groups were observed for LIMeco, with groups 2, 3 and 4 showing comparatively higher water quality than other groups.

Few statistically significant differences were observed for catchment area (only group 6 was different from the others). Slope of the river (not showed) gave the same differences of catchment size. Altitude differentiated groups 2 and 6 from others. Mean rain variability and mean temperature were not significantly related to AP clustering. Differences were found considering rain and temperature standard deviation (sd). Temperature sd exhibited significant differences between group 6 and all the others. Rain sd had the higher variability in group 2 that is the most diverse from other groups being composed of heterogenous river types including a relevant number of glacier-fed rivers and temporary streams.

The variability of selected large-scale anthropogenic pressures is reported in Fig. 5. Groups 1 and 6 were characterized by high levels of nitrogen wet deposition and groups 6, 7 and 8 by high median values of highly impacting agricultural land use. Groups 1, 6 and 8 had higher nitrate concentration when compared to other groups. Group 7, even if not significantly different from other groups, is positioned in the PCoA space close to groups 6 and 8 (Fig. 2). In group 1, all other anthropogenic pressures indicators were low. Group 5 exhibited the highest median artificial land use, accompanied by a certain degree of impacting agriculture, and had also high NH_4-N concentration.

In summary, AP groups—ordered by increasing water quality—showed a negative association with an overall

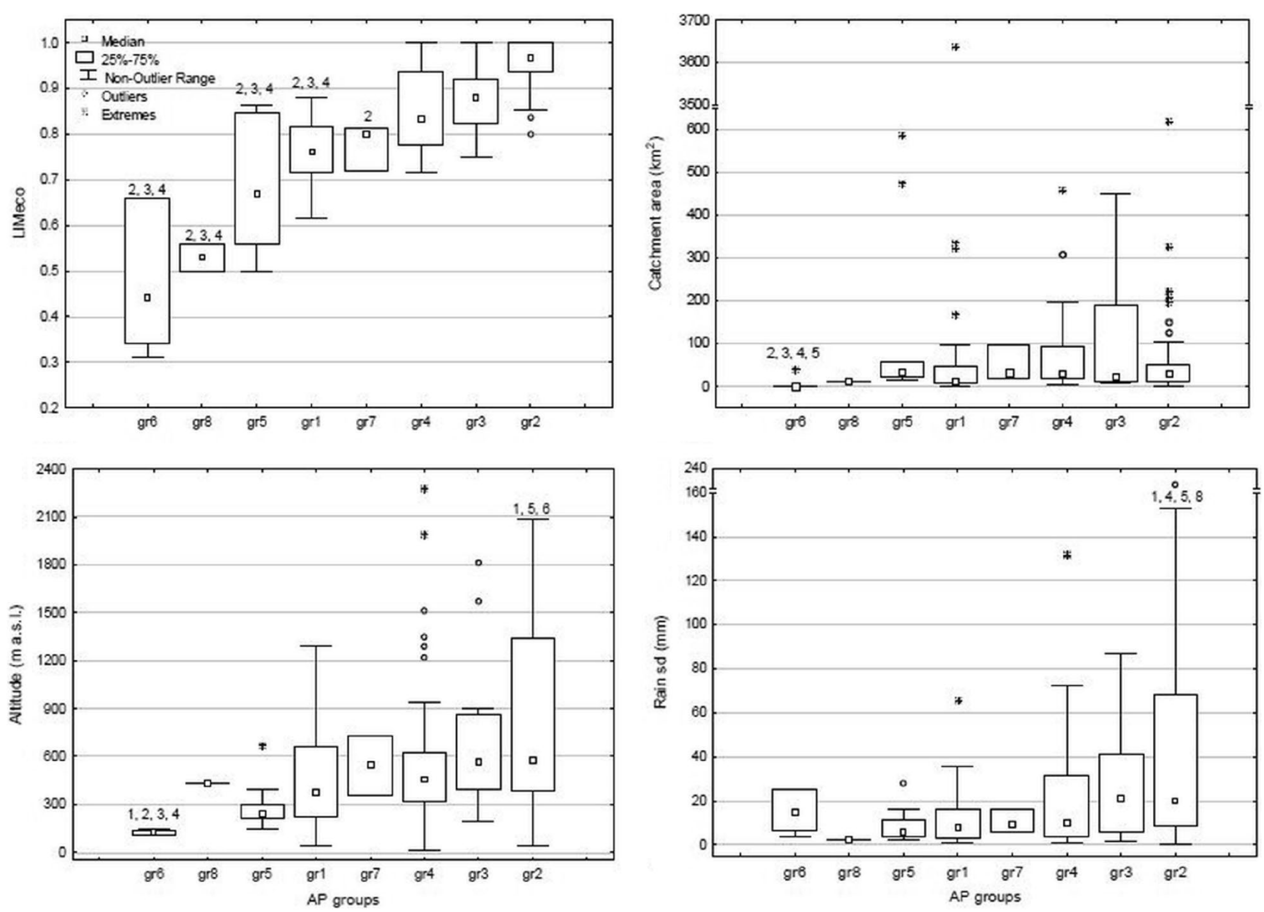

Fig. 4 Examples of water quality (LIMeco), typological and climatic features variability in the different AP clusters. Groups are ordered by increasing water quality (from left to right). Numbers represent between-group differences by Dunn's test

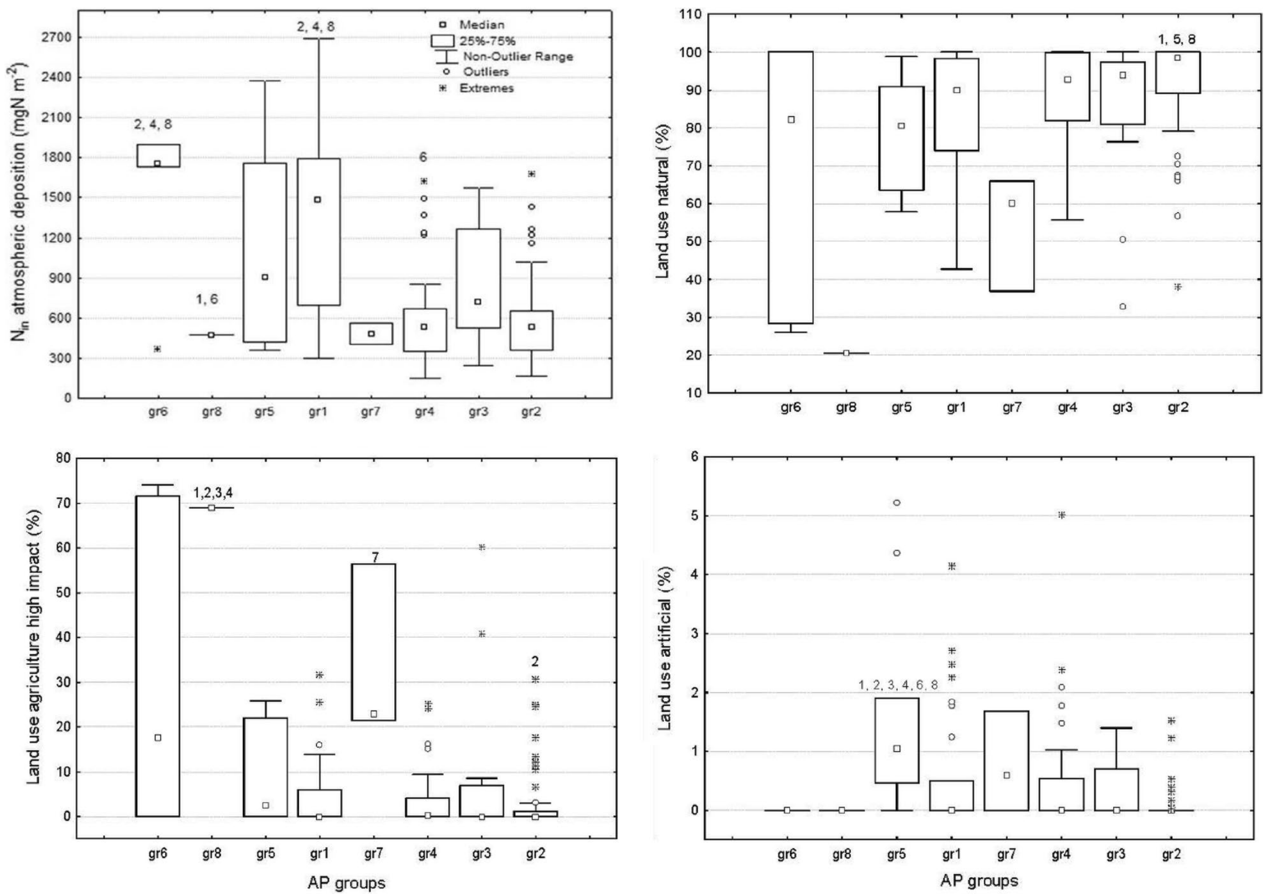

Fig. 5 Range of large-scale anthropogenic pressures variability in the AP clusters ordered by increasing water quality (from left to right). Numbers represent between-group differences according to Dunn's test

left-to-right reduction of non-natural land use and/or nitrogen deposition.

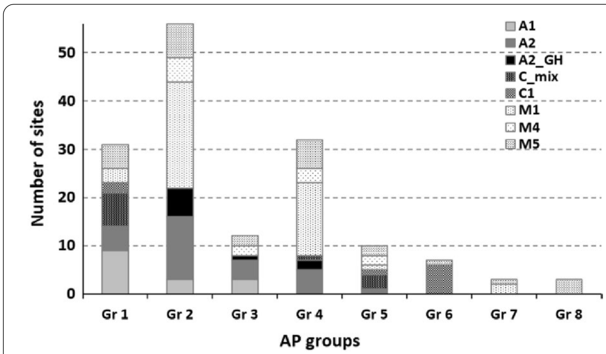

Fig. 6 Sites distribution among AP groups

Environmental conditions and the existing river macro-types

Figure 6 shows that sites belonging to different macro-types were evenly distributed among the eight AP clusters. Exceptions were the 6 and 8 AP clusters, assembling sites typically belonging to stream type C1 and M5, respectively. However, M5 sites are almost uniformly distributed among AP groups. In summary, AP clustering results did not match with the existing river macro-types defined for the EU WFD purposes.

Descriptive features of the exemplar elements of the AP groups (Additional file 1: Table S6) further confirmed that groups identification was guided from anthropogenic factors, more than typological ones (e.g., altitude, catchment). Figure 7 shows some of the exemplar reference sites according to the group they belong to, emphasizing that similar featured sites fit in different groups (e.g., AP group 3 and AP group 2).

Lowland sites (macro-types C1 and C_mix) were exemplar of two different AP groups (6 and 5). Exemplar sites of three different AP groups (2, 3 and 4) were alpine high-altitude sites (macro-types A2 and A2_GH). None of the exemplar sites technically belong to the medium-sized category (> 150 km^2), all having small- to very small-sized

Fig. 7 Exemplar sites from selected AP groups, ordered by increasing water quality of the group they belong to. AP group 6 (top, left): comparatively high nitrogen atmospheric deposition; AP group 5 (top, right): artificial land use in the catchment sometimes above reference site thresholds; AP group 3 (bottom, left): natural land use dominant in the catchment; comparatively high oxygen saturation levels; AP group 2 (bottom, right): natural land use largely dominant in the catchment, nutrients concentration very low (best observed reference sites)

catchments. Exemplar sites of four groups (1, 5, 6 and 8) were characterized by higher values of NO_3-N. The exemplar site of group 5 also showed high concentration of NH_4-N. Exemplar sites of AP groups 1, 5 and 6 showed high nitrogen wet deposition even in presence of a high level of natural land uses in the catchment (> 95%). High NO_3-N of Group 8 exemplar site was associated to comparatively high levels of agriculture with high impact. The exemplar site of AP group 7 had a relatively low oxygen saturation in combination with a comparatively low percentage of natural land use (see also Figs. 2 and 3). Lastly, the exemplar sites of AP groups 4, 3 and 2 had very low nutrients values.

Discussion

What nutrient levels might naturally be expected?

We analyzed reference sites over a wide latitudinal and climatic range including high elevation alpine basins and areas with typical Mediterranean climate. Altitude of the considered sites varies from 20 to 2280 m asl, the average annual rainfall from 400 to 1800 mm and air temperature from -1 to 17 °C. To cope with the expected diversity of riverine biological communities in this broad geographical context, the Italian legislation [43] defined more than 100 river types (sensu WFD) grouped in 8 macro-types [27]. What is the expected concentration range of nutrients in such a wide climatic and geographical gradient? Characteristics such as climate, hydrology, natural vegetation and mineral composition of soils and rocks were recognized as factors able to explain the variability of nutrients between river sites in natural areas [7]. Temperature controls the biological activity in soil influencing the rates of both N mineralization and nitrification and, ultimately, the NO_3 leaching from soils to surface water [8]. Annual runoff (stream discharge per unit drainage area) was identified as a strong predictor of total nitrogen (TN) load of pristine streams from American tropics and the Gambia River basin (Africa) [44]. Because specific runoff is related to precipitation, vegetation and soil moisture, it may reflect a complex of abiotic and biotic factors affecting nutrient transport and potential for biological retention. If the weathering of P-rich bedrocks is well recognized as possible source of P in stream waters [45–47], less evidence is available to demonstrate the influence of geological substrate on N. For instance, Holloway et al. [48] showed that certain metasedimentary and metavolcanics rocks (e.g., phyllite and slate), which contain appreciable concentrations of nitrogen (inorganic and/or organic forms), contribute a relatively large amount of nitrate to surface waters in some California catchments.

In natural basins most studies revealed very low nutrient concentrations, typically NO_3-N < 0.2 mg l^{-1}, NH_4-N < 0.02 mg l^{-1}, PO_4-P < 0.01 mg l^{-1} and TP < 0.02 mg l^{-1} [9, 10, 44, 49]. Therefore, the expected nutrient variability due to environmental factors in natural waters is commonly constrained within these upper limits. Considering the nutrient median values found in Italian reference sites, it is evident that NH_4-N is in line with such expected thresholds, TP is slightly above, and NO_3-N is twice the limit. In addition, the upper percentiles, especially for NO_3-N, largely exceed the above cited thresholds. For many river types, reference sites in natural conditions can hardly be found in industrialized and urbanized regions all over the world. Table 4 reports the nutrient average and/or relevant percentiles derived from reference sites in different countries including the values obtained in the present study. It is evident that in most cases, in the dataset here analyzed, the nutrient concentrations are higher than those reported for river stretches in pristine conditions (i.e., natural sites, unpolluted or anthropogenically undisturbed). TP and NH_4-N values observed in our dataset are fully comparable to those found in other EU countries and outside Europe. In contrast, Italian sites showed nitrate concentrations higher than other Mediterranean countries (particularly Greece) and Montana (USA) and closer to European Baltic-Central regions. Furthermore, there is a paucity of data on

Table 4 Nutrients and oxygen values found in the literature for reference sites, covering a global geographical gradient with a focus on Europe and Mediterranean countries. Information is presented by continent/country

Rationale	Geographical Area	TP (mg/l)	NO_3-N (mg/l)	NH_4-N (mg/l)	DO %	Reference
Median of reference data	USA (all states)	0.013–0.048	na	na	na	[76]
75th percentile of reference data	USA (Montana)	0.003–0.170	0.01–0.25	0.004–0.09	na	[78]
80th of least disturbed condition	Canada	0.011–0.062	na	na	na	[81]
75th percentile of reference data	Northeast China	0.046	na	na	na	[82]
Thresholds for environmental Class I quality standards for surface water	China	0.020	na	0.15	na	[83]
Rough values at 4 least disturbed sites	Australia (New South Wales)	0.033–0.077	na	na	94.5–97.8	[84]
Mean reference data	Europe (Central- Baltic)	0.020–0.040*	2–6	0.05–0.10	95–105	[20]
90th percentile of reference data	Europe (Mediterranean)	0.070	1.15	0.09	73.7–127.9	[19]
75th percentile of reference data	Europe (IT, UK, FR, CY)	0.050	2.82	0.04	100	[18]
90th percentile of reference data	Finland	0.015–0.020 (0.040**)	na	na	na	[47]
Reference thresholds based on predictive models	Sweden	0.025 (0.030**)	na	na	na	[47]
Natural and semi-pristine conditions derived from expected output rates from the catchment (mean values)	UK	0.006–0.493	na	na	na	[10]
High-quality thresholds (very low anthropogenic influence)	UK	0.020*	1.1	na	na	[85]
75th percentile of reference data	Cyprus	0.005	0.1	na	105	[86]
75th percentile of reference data	Greece	0.0865	0.18	0.016	na	[87]
75th percentile of reference data	Spain	0.082*	0.672	0.038	98.9–116.4	[88]
Reference thresholds values	Portugal (Mondego basin)	0.060	0.67	0.1		[89]
75th percentile of reference data (90th for TP)	Italy (state wide, all river types)	0.050	0.60	0.03	90	[27]
75th percentile of reference data	Italy (state wide)	0.049	0.88	0.03	100	This paper
90th percentile of reference data	Italy (state wide)	0.060	1.39	0.05	104	This paper

With few exceptions explicitly described, values are based on statistical descriptors of reference site data distribution. In some cases, values are generically referred to high quality or undisturbed river sites, not precisely to reference sites. Ranges are reported when different values are considered in different river types or ecoregions.

* P-PO4

** Lowland clay-rich rivers

na: not available

inorganic N forms because many countries (e.g., USA) prefer to use TN for monitoring, which is more easily related to biological elements, especially algae [11, 50].

The influence of anthropogenic factors on nutrient levels in the studied reference sites

The results obtained from our analysis on Italian reference sites demonstrate that the major factors determining differences in nutrient concentration were atmospheric deposition of inorganic N and presence of artificial and/or high-impact agricultural land use in the upstream catchment.

It is well known that the ubiquitous presence of anthropogenically enhanced N deposition has altered the N supply to many basins otherwise undisturbed [51, 52]. Several studies have associated changes in surface water chemistry, particularly NO_3 variations, with increased N deposition in natural forest catchments [8, 53–55]. The N_{in} deposition loads that characterize the basins analyzed in the present study ranged from 150 mg m^{-2} y^{-1}, not far from the expected level (i.e., 50 mg m^{-2} y^{-1}) in the absence of human influence, to values > 2000 mg m^{-2} y^{-1} (max = 2690), that are often related to a critical degree of N saturation of ecosystems [53, 54]. The highly significant correlation (Pearson's r = 0.60, $p < 0.0001$) between water NO_3 concentration and N_{in} deposition loads indicates the important role of atmospheric long-range transport of N compounds in influencing the oxidized N content in the studied water bodies.

Rain amount contributes to form the deposition load, and a positive relation between these two variables is evident for our sites in rain amount ranging from about 500 to 1300 mm per year (Additional file 1: Material S7). At higher rain values the relation is reversed. This means that for the Mediterranean sites (e.g., the majority in the AP groups 2, 4 and 8), characterized by a comparatively dry climate, the N atmospheric deposition does not constitute a significant alteration factor. The same is true for those sites characterized by the highest rain amount and located at the highest elevation (1650–2200 m asl), being thus relatively protected from long-range atmospheric pollution (e.g., present in the AP groups 2 and 4). For the sites with intermediate levels of rain, multiple anthropogenic factors contribute to increase the rain N concentration and thus to form the higher N deposition loads. The closeness to the industrialized and urbanized areas [56] is very relevant in this regard, along with the agriculture areas, which are sources of NO_x due to fertilizer use [57]. Several studies demonstrated the importance of atmospheric deposition in affecting the nutrient concentration in relatively undisturbed river sites. Lewis et al. [44], analyzing 20 minimally disturbed watersheds of the US, has found that atmospheric deposition was a significant predictor of nitrate, ammonium, and dissolved organic N in the waters. Smith et al. [7] demonstrated that the performance of a predictive model for TN and TP yields in 63 minimally impacted US rivers can be significantly improved if atmospheric deposition is included among the explanatory variables. They also estimated that the contribution of atmospheric deposition to background total nitrogen yield and concentration was higher than 40% in numerous nutrient ecoregions.

The fraction of basin occupied by highly impacting agricultural crops (e.g., maize), in addition to atmospheric deposition, plays a role in controlling NO_3-N concentration in Italian reference sites. It is widely recognized that nitrate is among the most important pollutants in waters impacted by agricultural areas where it is leached from the soils treated with organic or synthetic fertilizers [1, 58]. The high-impact agriculture has a high potential risk of nitrate leaching as great amounts of N fertilizers and irrigation water are applied to achieve the maximum yield crops [59]. Recently, Balestrini et al. [60] demonstrated that the percent of soil cultivated with maize is an effective factor in controlling the NO_3-N concentration in an Italian river type, named *fontanili*, also included in our data set. These water bodies, very common in the Po Plain, are small lowland streams completely fed by groundwaters [60, 61], which are seriously impaired by nitrate contamination [62]. This groundwater pollution is pervasive and often affects large areas, also due to groundwater circulation and long time for recovery. In some respects, however, *fontanili* represent the latest vestiges of the natural environment previously present in the northern Italian plains and they provide habitat for, and host animal species of community interest [60]. For these reasons, the more unaltered ones are usually accepted as reference sites despite their relatively elevated concentration of nitrate (mean ± sd: 1.90 ± 1.35 mgN l^{-1}) and the presence of highly impacting agriculture in adjacent lands (mean ± sd:14.1 ± 22.5%). The inverse relationship between NO_3 and oxygen saturation, both defining the first axis of the PCoA analysis, is partially explained by the presence of these waterbodies fully dependent from groundwater (GDE's, groundwater-dependent ecosystems, [63]), being thus characterized by a comparatively low oxygen concentration.

NH_4-N and TP concentrations, were related to the presence of agricultural land use and also to the presence of artificial land uses in the catchment even if its proportion was < 2%. The sources of NH_4 and TP in surface waters are not different from NO_3 (diffuse and point-sources), but the different chemical characteristics—NO_3 very mobile contrary to NH_4 and TP that have higher affinity for solid phases—influence the transport and fate of these nutrients. The AP group 5, which aligns along the second axis of the PCoA analysis, is characterized by the highest observed concentration of NH_4-N, high TP and a relatively high fraction of artificial land use. The group is composed of sites at low–medium altitude (150–391 m asl, with one exception) mostly located in the valley floor where some urban settlements surrounded by nearly natural catchments can be found. These features suggest the presence of occasional unauthorized discharges, requiring further investigations aimed at potentially exclude such stretches from the list of reference sites. In agreement with our results, [64] and [65] reported that even a low percentage of urban areas in the catchment can affect TP concentration. It is important to note that the fraction of basin covered by low-impact agriculture was not significantly related to PCoA axes and to AP clusters. This suggests that the presence in the catchments of small areas (quartile range: 0–11%) occupied by pasture and by a mix of agricultural patches and natural vegetation did not significantly influence the concentration of N and P in stream waters.

Some natural factors such as rain and temperature variability (standard deviation), elevation, catchment size and slope were significantly related to the PCoA axes and showed different values between AP clusters. This relationship might be explained as a covariation with the anthropogenic factors as in the case of catchment size in opposition to atmospheric deposition loads (Fig. 3). The small lowland streams of the Po plain (i.e., *fontanili* type) are characterized by very small catchments and high N deposition load being located in areas with intensive agriculture land use and close to large urbanized areas.

In general, the anthropogenic features discussed above determined a gradient in water quality among the studied reference sites, largely determined by comparatively high nitrate concentrations and summarized by the LIMeco descriptor, which was strongly associated to group distribution. This connection is partially expected because the LIMeco calculation is based on nutrient (including nitrate) concentration and oxygen saturation. Its formulation was envisaged to quantify nutrient-related overall impact on water quality and it varies only if each nutrient concentration exceeds predetermined thresholds (see Additional file S2). The gradient observed for this descriptor, which depicted the major nutrient trend across reference sites, attests that even where anthropogenic influence is low LIMeco is able to effectively summarize water quality degradation.

Selecting reference sites and the challenge of defining reference conditions for nutrients

Identifying reference conditions is key to environmental management and monitoring of rivers in Europe and worldwide [66]. Ecological classification is often measured as degrees of deviation from reference conditions and globally, the most consolidated classification schemes are based on regionalization and typologies [67–69]. For a river type or group of types, reference sites should represent pristine or non-impacted river reaches. However, the concept of 'least disturbed' site is often applied in the selection of river reaches to define reference conditions [12, 19]. The results of the present study demonstrate that, in most cases, factors linked to anthropogenic pressures are responsible for nutrient concentration in reference sites. In fact, the term 'reference site' is often linked to the concept of 'acceptable level' of anthropogenic disturbance [20, 70]. In Italy and many other European countries, the procedure to select reference sites requires the examination of a large set of features to accept a site as a reference [19, 25]. The simultaneous check of multiple criteria, which have to satisfy qualitative or quantitative requirements, determines the acceptance or refusal of a site as reference. For instance, criteria for land use are set considering lower or upper limits for reference site acceptance, e.g., artificial land use lower than 1% and intensive agriculture lower than 20% (and never in the proximity of the river channel). According to different geographical contexts (e.g., Mediterranean vs central European rivers, lowland vs mountain rivers) acceptance levels can differ [19, 20]. In general terms, nutrient information is included among criteria to validate the choice of reference sites, especially by setting maximum acceptable limits for each representative nutrient. Generally, a few thresholds for individual criteria can be exceeded if additional more stringent criteria are verified (% of agriculture can exceed limits if, e.g., there is no visible erosion in the catchment). When speaking of reference conditions, it is commonly accepted that anthropogenic effects on the environment cannot be undetectable, at least not everywhere, if we consider human beings as part of the biota [68]. Therefore, some areas are more susceptible to their anthropogenic history [68]. Accordingly, in the USA land use is one of the variables, among others that are more strictly related to natural characteristics, defining ecoregions and higher or lower levels of nutrient concentration can be accepted for reference sites [71]. In the present study, two groups of reference sites (6 and 8) were characterized by comparatively high nutrient concentrations and by a certain degree of agricultural land use. These groups include lowland sites located in two of the most exploited Italian areas, i.e., the Po plain (Northern Italy) and the Tavoliere (Puglia, South Italy). Additionally, sites of group 6 have origin from groundwater, known to be rich in nitrate originating from anthropogenic sources. In such situations, the criteria used to select reference sites possibly allowed for some least disturbed sites to be accepted as reference [12]. Excluding the aforementioned exceptions, which can be identified, circumscribed and described, the overall selection procedure used in Italy seems sufficiently adequate. In fact, sites from areas where human presence is not so pervasive showed low nutrient concentrations, in agreement with expectations.

We also demonstrated the key role of long-distance atmospheric deposition of nutrients. To our knowledge, this factor is not commonly included as a parameter to be monitored to assess reference conditions, at least in Europe. There is the need to carefully consider the fact that in certain basins or regions nitrate concentration in rivers can be high due to atmospheric deposition. In such situation it might be beneficial to establish higher NO_3-N thresholds in order to accept a site as reference site. The possible influence of atmospheric deposition should not lead to an acceptance of generally elevated levels of N everywhere. In fact, some areas, e.g., southern Italy, are less affected by this pressure (see "The influence of anthropogenic factors on nutrient levels in the studied reference sites"). In such context, the AP cluster analysis identified some exemplar sites that have a germane meaning in the context of the official network of WFD reference sites in Europe and may stimulate future investigations and refinements. In particular, exemplar site characteristics can guide the selection of further reference sites and support a better comprehension of processes acting at different areas and influencing nutrient concentration in freshwaters.

Looking beyond the horizon: from ill-defined reference conditions to adjusted nutrient thresholds for ecological status evaluation?

We observed that nutrient concentrations at reference sites sometimes exceed the narrow limits of expected natural nutrient variability (see "What nutrient levels might naturally be expected?").

Our study does not support the use of the current typological approach to define reference state with respect to nutrients, at least in Italy. We demonstrated that typological factors (e.g., catchment size, river regime) did not play a major role in clustering river reference sites and AP grouping did not indicate differences in nutrient concentration between river macro-types. This fits with the overall impression—described in Europe by Phillips et al. [72]—that there is little evidence of any clear river type-specific differentiation in nitrogen and phosphorous concentrations. This contrasts with the general feeling that an approach based on river types or macrotypes is somehow expected also to assess nutrient concentration variability [73]. There are, however, far fewer specific nutrient concentration thresholds than numbers of national river types because, usually, different types are referred to common concentration values [11]. With explicit reference to the Italian situation, the actual legislation [27] demands the use of the LIMeco descriptor—which is based on nutrients concentration and oxygen saturation—to classify water quality in rivers when defining the overall ecological status. At present, the LIMeco classification centers on scores assigned on the basis of thresholds derived from pooled samples collected at reference sites from all over the country. The used scores are thus nation-wide and not type specific, and the used approach fits with the findings of the present paper, apart from NO_3-N, which might deserve a regional adaptation (see below). Anyhow, once nutrient values are attributed to range classes, transformed into scores and combined, like in the LIMeco descriptor, the regional differences observed for nutrients in reference sites seem not to impact on the legally binding classification of water quality in Italian rivers.

In more general terms and from a management perspective, we cannot forget that at European level biological assessment methods have to be formally intercalibrated in order to settle a common perception of 'good' and 'high' ecological status. In such a context, how can we complement the definition of ecological status by integrating a WFD-compliant and agreeable approach to the use of nutrient information? When speaking of nutrient-related topics, the overall framework is complex and scattered. There is a clear limitation linked to the parameters that are effectively measured and a lot of discrepancy between countries is observed. The definition of nutrient levels to support the evaluation of ecological status based on the direct link between nutrients and the response of biological communities is strongly encouraged [73]. However, this is still not widely applied. In fact, expected non-linear responses between nutrients and ecological status [6], jointly with the common presence of multiple pressures in rivers, make it complex to model the risk of failing environmental objectives [74]. Recently, [75] tried to investigate such relationship in five river types in Europe, finding reliable relationships between nutrients concentration and biological communities in only two of them. In this context, the use of a fixed percentile in reference sample frequency distribution can be proficiently applied to any national dataset to derive thresholds for single parameters, independently from the measured parameters and from the relationship with the biota. Such an approach is widely used to define benchmarks for biological and/or abiotic data [18, 76–78].

We revealed that when differences are found between river reference sites, they are mainly linked to hidden anthropogenic factors that control nutrient levels in Italian reference sites located in geographical areas where pristine conditions are not available anymore. In these areas, a comparatively high degree of either land use modification, high nitrogen deposition rates, groundwater NO_3-N contamination or their combination are observed. Therefore, the key step for setting nutrient thresholds is still the validation of reference conditions. In this regard, we suggest the use of regional adaptations based on the concept that baseline human effects can be accepted in some geographical areas when selecting reference sites. This will not only allow to schematize and classify the complexity of ecosystems within their human background, but it would also emphasize the need for a compromise for a more effective management and assessment of the environment [68]. Such a solution should be based on detailed analysis, to avoid a generalized acceptance of inadequate quality at reference (best available) sites. This has to be coupled with the need for regional studies to better evaluate the vulnerability of particular ecosystems like GDEs [63] and headwaters [79]. If lower quality reference sites have to be used, they should at least be categorized separately to make explicit this distinction as proposed in [12]. When low-quality sites are included among reference, the difference between reference and non-reference sites would be reduced, thus precluding a correct quantification of anthropogenic stress [69]. In situations where only least disturbed sites can be found, other approaches (e.g., dose–response) might be used to assess the level of confidence of such sites.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-022-00642-y.

Additional file 1: Table S1. Investigated sites and selected geographic features. **Table S2a.** Parameters thresholds and scores assigned for LIMeco calculation (Buffagni et al., 2008; DM 260/2010). **Table S2b.** Thresholds for LIMeco classification. **Table S3.** Summary statistics for the selection of the AP solution and number of groups. **Material S4**. Association between environmental, physicochemical and anthropogenic variables and PCoA axes. **Material S5**. Complete results of BDM and Dunn's tests. **Table S6.** Mean values of selected variables in exemplar sites of Affinity Propagation (AP) clusters . **Material S7.** Variability of atmospheric deposition rates of inorganic nitrogen ordered by increasing yearly rain amount 200.

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Author contributions

SE, AB and RB: conceptualization, investigation, formal analysis, data curation, visualization, writing—original draft, review and editing. MC: investigation, data curation writing—review and editing. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to the fact that part of the data are research data derived from complex datasets, still not fully published. Part of the data were also collected by different water authorities and partly available on the Informative System for Water Protection SINTAI—a national repository containing data on water bodies collected by Italian Environment Agencies. All the data are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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