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# Refinement of the NISECI ecological index reference conditions for Italian freshwater fish communities in the eastern Emilia-Romagna region

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ABSTRACT

Following the Water Framework Directive 2000/60/CE (WFD), each member state of the European Union must monitor compliance of its rivers with ecological quality standards through biological quality indicators. The New Italian Index of the Ecological State of Fish Communities (NISECI) was developed in 2017 for the assessment of fish communities, as directed by the WFD in Italian freshwater habitats. According to the WFD, the general reference conditions (GRCs) of NISECI must be refined on a regional scale through new calculation of its metrics and sub-metrics. In the present study we used environmental and ichthyological data from 457 fish samplings distributed among 299 sampling sites within 84 different water bodies collected from 1995 to 2012 to develop: 1) new lists of expected species for six homogeneous zones identified in the Reno basin (Italy) and in the eastern regional basins of the Emilia-Romagna region; and 2) the threshold values for their species-specific abundance. Results were set as refined reference conditions (RRCs) for two of the metrics used in the application of the NISECI index in the study area (i.e. $X_1$, relating to indigenous species and $X_{2,b}$, for the abundance of expected species). The RRCs were tested by applying NISECI to 24 monitoring sites of the regional surface water monitoring network (i.e., ARPAE) and comparing the results with the application of NISECI using the GRCs. Furthermore, the analytical power of the refined NISECI was evaluated by relating the findings to three expert-based blind assessments of fish community ecological status. The results confirmed an increase in refined NISECI values and its higher consistency with expert-based assessment, supporting the validity of the presented method for RRC development and its potential for application in other regions.

## 1. Introduction

Freshwater ecosystems are among the most modified environments in the world, facing severe anthropogenic impacts on biodiversity (Malmqvist and Rundle, 2002). Significant threats such as water pollution and invasion of non-native species are causing subsequent damage to freshwater biodiversity (Reid et al., 2019; Britton, 2022). Additionally, modifications to natural water bodies by structures such as dams and other intensive land-use changes have significantly impacted freshwater communities (Malmqvist and Rundle, 2002; Vié et al., 2008). In 2000, the European Union established the Water Framework Directive (WFD) aiming to preserve all aquatic habitats (Water Framework Directive, 2000). The main goals of the WFD include focusing efforts on assessing the quality of European rivers while also developing proper management strategies for the preservation of these water bodies. The WFD uses phytoplankton, macrophytes, benthic invertebrates, and fish as biological quality elements to evaluate the ecological status of freshwater systems (Water Framework Directive, 2000). Using fish has a wide array of advantages for monitoring programs. These include the extensive knowledge of fish life history, their representation within most trophic levels, the relative ease of species identification, the general public's relation to fish community health, their presence in most water bodies, and the possible evaluation of their levels of acute toxicity and stress (Karr, 1981; Whitfield and Elliott, 2002; Pinna et al., 2023).

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Anthropogenic impacts on freshwater communities such as habitat alteration, flow regime modification, pollution, and the release of alien species lower the diversity and abundance of native species (e.g., Karr, 1981; Fausch et al., 1990; Aparicio et al., 2011). Thus, fish quality indices provide an important tool for water quality assessment through the detection of disturbances caused by both the physical–chemical environmental changes and the hydrological regime of a lotic system (Sapounidis et al., 2019; Fornaroli et al., 2020).

The first freshwater fish index was developed in response to the Clean Water Act of 1977 (USA), which called for a more refined approach to the monitoring of water resources (Karr, 1981). Since its development, several new indices have been proposed for freshwater systems, such as the Index of Biotic Integrity (IBI) for characterizing fish populations and the ecological quality of Flandrian water bodies (Belpaire et al., 2000; Breine et al., 2021); the Fish-Based Index (FBI) for the assessment of river health in France (Oberdorff et al., 2002); Preliminary Multimetric Indices (PMI) to assess streams in Romania (Angermeier and Davideanu, 2004); the European Fish Index (EFI), which uses ecological characteristics of fish population to infer ecological status (Pont et al., 2006); the Jucar Index of Biotic Integrity (IBI-Jucar) to assess biological integrity of the Jucar river basin in the Iberian peninsula (Aparicio et al., 2011); the Fish-based BalkaN Biotic Index (fBNBI) to assess the ecological status of lotic systems in Serbia (Stojković et al., 2011); and the Fish-based River Integrity Index (FRII) for the assessment of rivers in Greece (Sapounidis et al., 2019). In Italy, The WFD is being implemented through the legislative decree 152 (2006) for maintaining an ecological policy geared toward sustainable development and integrated water resource management. Furthermore, a new Italian index of the ecological state of fish communities (NISECI; Macchio et al., 2017) has been established to analyze the quantitative and qualitative composition of fish populations in water bodies by using the ecological status of fish communities as a bio-indicator. The NISECI evaluates the general condition of an observed fish community based on three metrics, which consider the presence of expected indigenous species, the biological condition of their population, and the presence of alien/hybrid species (Macchio et al., 2017; see Materials and Methods for details). Based on the three metrics, NISECI assigns to the sampled river stretch one of the five possible categories of ecological status, ranging from "high" to "bad" (Macchio et al., 2017).

Most ichthyological indices, including the NISECI, measure the deviation of observed fish populations from a reference condition. Using a reference condition as a benchmark for metric scoring, however, can be difficult and unrealistic for management (Tweedley et al., 2017), especially under rapid environmental change that can alter the structure of pristine aquatic communities (Latli et al., 2017). Additionally, many countries lack pristine sites and national methods for defining reference conditions vary greatly (Jepsen and Pont, 2007). These challenges are further exacerbated since a key requirement of the WFD is that the reference conditions, in which all expected indigenous species are present, populations are in good biological condition, and no alien species or hybrids exist (equivalent to high ecological status), must correspond to the absence or minor presence of anthropogenic changes (Water Framework Directive, 2000). In Italy, riverine habitats are compromised in such a way that the identification of pristine reference sites is practically impossible, except for a few types of high mountain environments, and even in these cases, they must be carefully evaluated (Rossi et al., 2022).

Understanding the characteristics of freshwater environments is fundamental for the sustainable use of freshwater resources under anthropogenic pressure (Bogardi et al., 2020). The improvement of monitoring practices is necessary to evaluate the effects of human impacts on water networks and promote the conservation of freshwater resources (Turak et al., 2017). The current reference conditions for NISECI, defined hereafter as general reference conditions (GRC), are provided on a national and ichthyological district basis (Macchio et al., 2017). The Italian territory is divided into three ichthyological regions on a zoogeographic basis (Zerunian, 2002; Zerunian et al., 2009; Macchio et al., 2017). Three fish zones are defined within each ichthyological region: the salmonid zone, the lithophilic spawners cyprinid zone, and the phytophilic spawners cyprinid zone, each with its own expected fish community. These community partitions are intended to be further refined with historical and bibliographical analyses of fish communities based on the current framework (Macchio et al., 2017; Ministerial Decree 260, 2010). Refinement is crucial for NISECI implementation to avoid under- or over-estimating the ecological status of water bodies (Rossi et al., 2022) and should address both the list of expected species and their demographic reference values (abundance and population structure). For each individual fish species in each individual water body, the following must be defined for the purposes of refinement (Rossi et al., 2021): (1) whether the species is autochthonous; (2) whether it should be expected in the local ecological context; and (3) the threshold values for associating quality judgments with the observed values of the metrics and sub-metrics related to the biological condition of indigenous and alien species.

Considering the lack of pristine habitats in Italy, it is necessary to adopt a modelling approach to refine the reference conditions within each Italian zoogeographical context. Fish communities and ecosystem components are subject to natural variation over time. To define reference conditions, therefore, the ichthyological and environmental data used must be representative of a suitable and functional period for the monitoring plan (Rossi et al., 2022). In the present study, an extensive dataset over a 16-year period was used to elaborate new lists of expected species for NISECI application in the Reno basin and in the Emilia-Romagna eastern regional basins (Italy). Threshold values for the species-specific abundance of the present fish species were calculated under a spatially based approach, in which the best possible conditions estimated in the geographical and ecological contexts of the water body are adopted as reference conditions (Rossi et al., 2021). Both lists of expected species and threshold values were set as refined reference conditions (RRCs) for NISECI application and compared with GRCs, with the objective of providing a reliable methodology to refine NISECI metrics at sub-regional scale throughout the Italian territory.

## 2. Materials and methods

### 2.1. The NISECI

The NISECI provides an evaluation of the general condition in an observed fish community based on three metrics, each ranging from 0 to 1 (Macchio et al., 2017; details in Table 1):

- $X_1$ = "presence/absence of the indigenous species expected for the specific geographical and environmental context (reference community)".
- $X_2$ = "biological condition of the population of indigenous species". $X_2$ is calculated based on two sub-metrics:
  - o $X_{2,a}$="population composition of age groups"
  - o $X_{2,b}$="demographic consistency"
- $X_3$ = "level of harmfulness of the alien/hybrid species present, ratio between the number of alien/hybrid and autochthonous individuals, and ratio between the number of autochthonous and alien/hybrid species".

### 2.2. Study area

The hydrographic network of the Emilia-Romagna region belongs entirely to the southern side of the Padano-Venetian ichthyogeographic district (Bianco, 1995). At drainage basin level it can be divided into a) the Po river basin and its tributaries from the Tidone river in the west to the Panaro river in the east, and b) basins that flow directly into the Adriatic Sea, from the Reno river to the Tavollo river, all located in the eastern regional sector. This study was conducted along the following

**Table 1**
New Index of Ecological Status of Fish Communities, NISECI; formulas and details for NISECI, metrics and sub-metrics are reported; classification intervals for NISECI, $EQR_{NISECI}$ and Ecological Status are reported; *see Macchio et al. (2017) for further details.

$NISECI = 0.1x_1^{0.5} + 0.1x_2^{0.5} + 0.8(x_1 \times x_2) - 0.1(1 - x_3) \times (0.1x_1^{0.5} + 0.1x_2^{0.5} + 0.8(x_1 \times x_2))$

| *Metric* | *Definition* | *Details* | | |
|---|---|---|---|---|
| $x_1$ | $= (1.2n_i + 0.8n_a)/(1.2m_i + 0.8m_a) x_1 \in [0,1]$ | $n_i$ = number of observed indigenous species of greater ecological-functional significance (Salmonidae, Esocidae, and Percidae)<br>$n_a$ = number of other observed indigenous species<br>$m_i$ = expected number of indigenous species of greater ecological-functional significance (Salmonidae, Esocidae, and Percidae)<br>$m_a$ = expected number of other indigenous species | | |
| $x_2$ | $= \sum_{i=1}^{n}(0.6 \times x_{2,a,i} + 0.4 \times x_{2,b,i})/n$<br>$x_2 \in [0,1]$ | $n$ = number of observed indigenous species<br>$i$ = single observed indigenous species<br>***Submetrics*** | | |
| | | $x_{2,a}$ = population composition of age groups | well-structured = 1<br>medium structured = 0.5<br>unstructured = 0 | Judgement assigned based on two criteria*:<br>*criterion A* - distribution of individuals among the size classes<br>*criterion B* - ratio between the number of adults and juveniles |
| | | $x_{2,b}$ = demographic consistency | equal to expected = 1<br>intermediate = 0.5<br>poor = 0 | Based on threshold values for the distribution of density frequencies at regional or smaller scales* |
| $x_3$ | = 1 if no alien species are present<br>= 0 if at least one well-structured population of a species in List 1 exists *<br>= 0 if the total number of alien fish is greater than or equal to the total number of indigenous fish (belonging to expected species)<br>= 0.5 ($a_{min}$ +$b$) in all other cases<br>$x_3 \in [0,1]$ | ***Submetrics***<br>$a_{min}$ represents the lowest value of " $a$ " within the observed samples:<br>$a$ = 0.5 in the presence of species in List 1* with a poorly structured population<br>$a$ = 0.5 if the total number of alien species in List 2* is greater than or equal to the total number of indigenous species<br>$a$ = 0.75 if the total number of alien species in List 2* is less than the total number of indigenous species<br>$a$ = 0.75 if the total number of alien species in List 3* is greater than or equal to the total number of indigenous species<br>$a$ = 0.85 if the total number of alien species in List 3* is less than the total number of indigenous species.<br>$i = i + ii + iii$<br>$i$ = proportion of alien species with well-structured population compared to the total number of alien species present × 0<br>$ii$ = proportion of alien species with an average structured population compared to the total number of alien species × 0.5<br>$iii$ = proportion of alien species with unstructured population out of the total number of alien species × 1 | | |

***NISECI Classes***

| **NISECI** | $EQR_{NISECI}$ **= (logNISECI + 1.1283)/1.0603** | **ECOLOGICAL STATUS** |
|---|---|---|
| 0.525 ≤ NISECI | 0.80 ≤ $EQR_{NISECI}$ | High |
| 0.322 ≤ NISECI < 0.525 | 0.60 ≤ $EQR_{NISECI}$ < 0.80 | Good |
| 0.198 ≤ NISECI < 0.322 | 0.40 ≤ $EQR_{NISECI}$ < 0.60 | Moderate |
| 0.121 ≤ NISECI < 0.198 | 0.20 ≤ $EQR_{NISECI}$ < 0.40 | Poor |
| NISECI < 0.121 | $EQR_{NISECI}$ < 0.20 | Bad |

seven basins all flowing directly into the Adriatic Sea (Fig. 1): Reno (basin size = 5.040 km$^2$), Lamone (basin size = 500 km$^2$), Fiumi Uniti (basin size = 1240 km$^2$), Savio (basin size = 625 km$^2$), Bevano (basin size = 94 km$^2$), Rubicone (basin size = 583 km$^2$), and Marecchia (basin size = 941 km$^2$).

### 2.3. Taxonomic, nomenclature and zoogeographic updates

Since the fish communities reported in the general formulation of NISECI are not based on a recent zoogeographic framework (Zerunian et al., 2009), it is necessary to confirm which species are autochthonous (i.e., indigenous, with respect to the zoogeographic context of application) prior to the application of the index for monitoring purposes. The list of species has been updated following the Italian inland waters autochthonous fish checklist, which was reviewed by the Italian Association of Freshwater Ichthyologists (AIIAD) and the IUCN Red List (Kottelatt and Freyhof, 2007; Freyhof et al., 2008; Freyhof, 2011; Bianco, 2014; Dyldin et al., 2017; Lorenzoni et al., 2019; AIIAD, 2022). The nomenclature used in this study for *Salmo* species followed the Italian freshwater fish taxonomy (i.e., *Salmo ghigii*) to allow a more precise and applicable functioning of the index for monitoring purposes, since the NISECI is specific for the Italian monitoring program.

### 2.4. Data source

Fish and environmental data were collected from 1995 to 2012 (Table S1) during regional projects for the conservation and management of fish species and EU WFD monitoring activities in collaboration with the regional agency for environmental protection (ARPAE). The dataset included 457 sampling activities of both fish community and environmental parameters, distributed among 299 sites in 84 water bodies where ichthyological data of all collected fish species were recorded. Data on the collected indigenous fish species (n = 16) are presented in the results, while details on alien species are not shown since they are used only for the calculation of the $X_3$ metric, which was not refined in this study. The altitude of sampling sites ranged from 2 to 1070 m of elevation. Spatial distribution of sampling sites had poor coverage at the lowest altitudes, where many water bodies are identified by national legislation (Legislative decree 152, 2006) as Heavily Modified Water Bodies (HMWB). However, the lack of data in this area does not affect the purpose of the study since the NISECI is not intended for the application at HMWB sites.

For each sampling site, altitude and average slope relating to the sampling stretch were evaluated in the GIS system (QGIS software version 3.10.14) based on the Emilia-Romagna regional technical map (1:5000). To determine the average slope of the sampling site, a pair of elevation points indicating the upstream altitude, downstream altitude, and length of the stretch was identified for each river stretch. The slopes were then calculated with the following formula:

$$slope(\%) = \Delta altitude \quad (m) \quad *100/length \quad of \quad the \quad stretch \quad (m)$$

Width was defined for each site as the average of three or more measurement points (depending on local variability) along the sampled river stretch, measured using either a meter rope or a laser distance meter.

For each waterbody, ichthyological data were associated with the typification of the riverbed in mesoscopic units (Forneris et al., 2007; Table 2). At the microscopic level, the lytic substrate of the riverbed was classified as Uncovered Rock, Boulders (>350 mm), Stones (100–350 mm), Pebbles (35–100 mm), Gravel (2–35 mm), Sand (1–2 mm) and Mud (AIIAD, 1996). Both mesoscopic and microscopic environmental

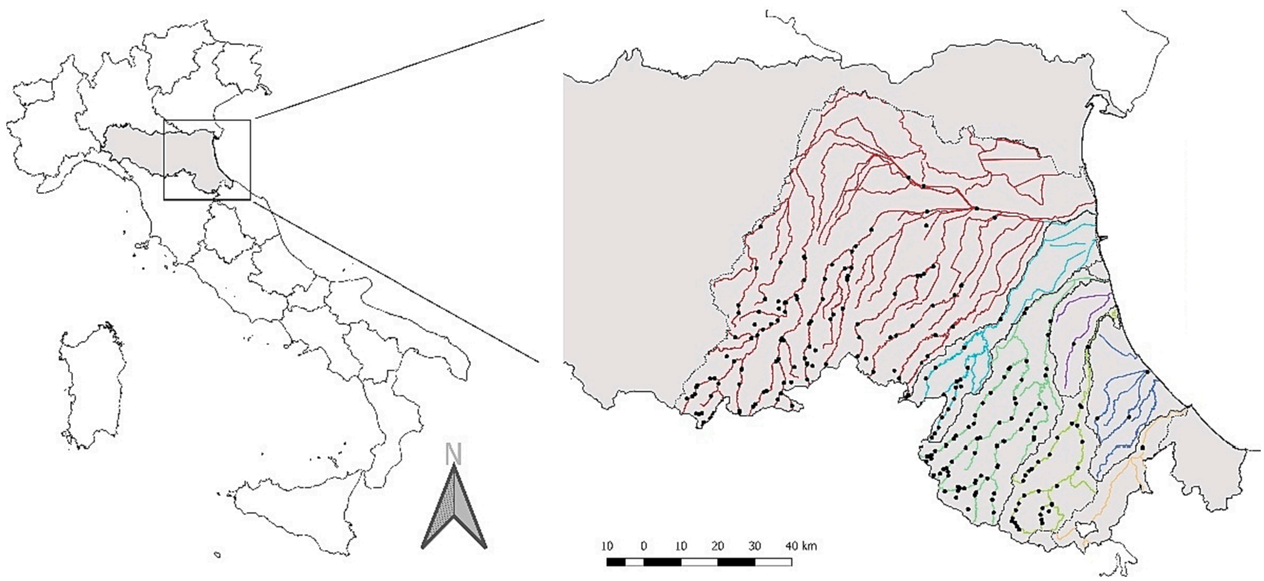


**Fig. 1.** Study area with indication of fish data sampling sites (dots). Basin networks of Bevano (purple), Lamone (light blue), Marecchia (yellow), Reno (red), Rubicone (blue), Savio (light green), and Fiumi Uniti (dark green) are indicated. Grey shading indicates the Emilia-Romagna region. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 2**
Mesoscopic environmental variables.

| **Mesoscopic environmental variables** | |
|---|---|
| *Waterfalls* | water flows over a vertical drop > 1 m high |
| *Cascades* | water flows over a vertical drop 0.5–1 m high |
| *Hops* | water flows over a vertical drop < 0.5 m high |
| *Riffles* | relatively uniform slope, where the water flows at high (usually on gravelly bottoms) or very high speed (usually on pebbly bottoms) with surface ripples and turbulence |
| *Pools* | depressions of the riverbed deeper than adjacent areas |
| *Runs* | homogeneous and constant current depth and speed (from 6 to 30 cm $s^{-1}$, more rarely up to 40 cm $s^{-1}$). Absent or limited surface turbulence |

variables were expressed as percent coverage of the total sampled surface. Descriptive statistics for environmental variables in the 457 samplings are reported in Table S2. These environmental variables were used to define homogeneous environmental typologies that represent particular zones, which was necessary to refine the GRC community to type-specific reference conditions.

The fish samplings were carried out by electrofishing according to the standardized protocols that have been used since the 1990s and were later formalized in a published protocol (Sollazzo et al., 2007). The sampling team was composed of five or more operators: one dedicated to the use of a straight DC electrofisher, three to the collection of the specimens and one or more to the transport of the specimens to the base camp. Most samplings were carried out with a single-pass catch and only a minor part with the multiple-pass removal method (Zippin, 1956, 1958; Teixeira-de Mello et al. 2014). After being provisionally placed in buckets filled with river water, the fish were transported to the base camp located near the sampling site, where individual total length was measured with a ruler (±1 mm) and weight was measured with a precision scale (±0.1 g). All indigenous species identified within the dataset are listed in Table 3.

Gross abundance values of fish samples on single-pass catch (number of collected specimens per species) were normalized to a Catch per Unit Effort (CPUE) per 100 m river length using the following formula (Mueller et al., 2018):

$$CPUE = \frac{number\ \ of\ \ specimens}{total\ \ lenght\ \ of\ \ sampled\ \ stretch\ \ (m)}*100$$

**Table 3**
Fish species present in the dataset. A reference code used for further analyses is reported for each species.

| Reference code | Order | Family | Species |
|---|---|---|---|
| AA | Cypriniformes | Cyprinidae | *Alburnus arborella* (Bonaparte, 1841) |
| AN | Anguilliformes | Anguillidae | *Anguilla anguilla* (Linnaeus, 1758) |
| BP | Cypriniformes | Cyprinidae | *Barbus plebejus* Bonaparte, 1839 |
| BC | Cypriniformes | Cyprinidae | *Barbus caninus* Bonaparte, 1839 |
| CB | Cypriniformes | Cobitidae | *Cobitis bilineata* Canestrini, 1865 |
| CC | Cypriniformes | Cyprinidae | *Cyprinus carpio* Linnaeus, 1758 |
| SS | Cypriniformes | Cyprinidae | *Squalius squalus* (Bonaparte, 1837) |
| PB | Perciformes | Gobidae | *Padogobius bonelli* (Bonaparte, 1846) |
| RB | Cypriniformes | Cyprinidae | *Romanogobio benacensis* (Pollini, 1816) |
| PG | Cypriniformes | Cyprinidae | *Protochondrostoma genei* (Bonaparte, 1839) |
| SH | Cypriniformes | Cyprinidae | *Scardinius hesperidicus* Bonaparte, 1845 |
| CS | Cypriniformes | Cyprinidae | *Chondrostoma soetta* Bonaparte, 1840 |
| CG | Scorpaeniformes | Cottidae | *Cottus gobio* Linnaeus, 1758 |
| SG | Salmoniformes | Salmonidae | *Salmo ghigii* Pomini, 1941 |
| LA | Cypriniformes | Cyprinidae | *Leucos aula* (Bonaparte, 1841) |
| TM | Cypriniformes | Cyprinidae | *Telestes muticellus* (Bonaparte, 1837) |

### *2.5. Statistical analyses*

#### *2.5.1. NISECI expected fish communities for metric $X_1$*

Homogeneous zones were first identified within the study area through the analysis of environmental variables. Subsequently, lists of expected species were determined for each zone.

Principal component analysis (PCA) was performed to reduce the dimensionality of environmental datasets while preserving most of the information on the variance and covariance of the original variables

(Joliffe, 1986). Considering the differing nature of environmental variables, three PCAs were performed using RStudio on: 1) the absolute values of Slope and Width; 2) the six mesoscopic variables; and 3) the seven microscopic variables. Altitude was excluded from this analysis because it was considered misleading, as the samples are distributed over different types of water bodies, from second and third level steep stream tributaries to large rivers in the valley floor. Thus, two samples can have completely different hydrological conditions and habitats at the same altitude, due to differing slopes along the hillside (e.g., for tributaries) and on the valley floor (for main rivers). For each PCA, the PCs explaining > 70–80 % of the variance were used for the following cluster analysis.

Hierarchical cluster analysis (HCA) was applied to the selected PCs to identify homogeneous zones based on environmental variability. The agglomerative hierarchical approach with average linkage method was used in RStudio to calculate cluster distances. The K–mean algorithm (which allows to select the number of clusters prior to the analysis) was preferred, since the purpose of the study was to increase the number of zones reported in the NISECI GRC (Everitt and Hothorn, 2011). The average linkage method was used to calculate the distances between clusters (Sokal and Michener, 1958). The detailed algorithm is implemented in the kRCLUSTER function of the PRIMER software (PRIMER-E, Ltd., Ivybridge, UK) used to apply the procedure to our data.

Differences in species composition among the homogeneous zones resulting from the HCA were identified with PERMANOVA (Anderson, 2005) using "zones" as a fixed factor. Before the test, CPUE data from single-pass catch were standardized to relative composition, then a square root transformation was applied to all values to reduce the dominant contribution of abundant species (Somerfield and Clarke, 1997). The similarity matrix was calculated with Bray-Curtis differences (Bray and Curtis, 1957), considering that species-station matrices present many species simultaneously absent in more stations (Scardi, 2001). SIMPER analysis (similarity percentages, Clarke and Warwick, 1994) was performed to test the consistently present species contributing to assemblage similarities. This technique is useful to understand which species are most responsible for the differences among zones (Guerra-García et al., 2006). These analyses were performed in PRIMER V6 software (PRIMER-E, Ltd., Ivybridge, UK).

#### 2.5.2. NISECI demographic reference condition for sub-metric X2,b

The assessment of demographic consistency in the NISECI is performed by assigning the estimated density (number of individuals per $m^2$) to one of three categories of abundance, which are defined based on threshold values for the frequency distribution of estimated density values at the regional or greater scale (Macchio et al., 2017). The threshold values used to separate the three categories of abundance are defined as the 1st tertile (cumulative percentage of the sample = 33 %) and the 2nd tertile (cumulative percentage of the sample = 66 %) of the species-specific frequency distribution taken as a reference for the application. Since the thresholds need to be defined at regional or basin scale, no reference values are provided (Macchio et al., 2017). Here, the estimated density values were reported using the data collected through the multiple-catch removal method (Macchio and Rossi, 2014), which allows the estimation of total abundances (Moran, 1951; Zippin, 1956, 1958; Teixeira-de Mello et al. 2014). The multiple-catch removal method was poorly applied before the publication of the ISPRA protocol, so it is often difficult to find enough species-specific data to derive the threshold values for estimated density. For the study area, the number of estimated density data selected for each species involved in further calculations ranged from 5, for the rarest species such as *C. taenia*, to 49 for more common species such as *S. ghigii*, *S. squalus* and *B. plebejus*. Due to the limited amount of data, threshold values for this study were derived by modelling the trend of the estimated density values, to obtain more reliable estimates (District Basin Authority of the Eastern Alps, 2021). The estimated density values and their percentile rank within the entire distribution interval were interpolated according to the Michaelis-Menten model as it gave the best fit at both low and high densities for all species. Analyses were performed in Past 4 Software.

#### 2.5.3. General and refined NISECI application and comparison

To understand the effectiveness of the RRCs, including both type-specific reference communities and sub-metric $X_{2,b}$ threshold values, 24 sites in the study area sampled in compliance with the ARPAE monitoring activities under the WDF between 2019 and 2022 were used (Table S3). NISECI was applied using both the GRC communities (Macchio et al., 2017) and the new RRC communities identified in this study. The GRC community was assigned to each sample using the administrative zoning of the regional inland waters carried out in 2005 (Piano Ittico Regionale, 2006–2010) where the regional river basins are divided into i) D Zone (salmonid waters), ii) C Zone (lithophilic spawners cyprinid waters) and iii) B Zone (phytophilic spawners cyprinid waters). The RRC communities were assigned according to the environmental classification. Since the NISECI GRCs do not provide threshold values for the $X_{2,b}$ sub metric, those calculated in this study have been used both for GRC and for RRC calculations.

Quality class frequencies of both NISECI applications were compared using Fisher's exact test (McCrum-Gardner, 2008; Bower, 2003) with Past 4 Software.

A comparison with judgments assigned on expert basis in a blind approach was conducted to evaluate if the refinement improved the index performance in assessing the ecological status (Pagani et al., 2021). Three ichthyologists with long and proven experience in the regional freshwater context were involved in the study and for each of the 24 sites were asked to provide their estimation of the NISECI quality class after being provided with:

- ichthyological data, including species-specific abundance values and length frequency distribution of 1 cm size.
- environmental data, including description at meso (type of flow) and micro (substrate) scale of river habitat, description of riparian zone and photographic material.

Each expert was not informed about the judgements of the other two participating experts. The consistency of expert judgements was first tested among the three experts, then the mean of their judgements was compared to the GRC and RRC NISECI using the Kolmogorov-Smirnov test.

## 3. Results

### 3.1. Metric $X_1$ refined reference condition

Based on the PCAs, the total number of fifteen environmental variables was reduced to nine principal components (PC), one geomorphic PC, four mesoscopic PCs and four microscopic PCs, accounting for 71 %, 88 % and 84 % of the overall variance, respectively, of the global model (Table S4).

HCA was performed with k values ranging from five to eight and clustering into six groups was found to be the most accurate after testing the differences in species composition among clusters through a PERMANOVA test. Fish assemblages were significantly different among zones (PERMANOVA, $P < 0.001$; Table 4) except for the zone pair 2 and 3. Five out of six homogenous ecological zones followed an upstream–downstream gradient (Zones 1, 3, 4, 5, and 6), while Zone 2 consisted of some sites in the Forlì-Apennines that have high values of uncovered rocks even though they are located at low altitudes. The frequency of samples within each of the six zones is shown in Table S5 while the distribution of each environmental variable for each zone is shown in Figure S1.

The results of the SIMPER analysis (Table 5) showed the differences in species composition within each zone: in zone 1, the only species was SG; in zone 2 and 3, SG contributed the most similarity (49 % and 30 %

**Table 4**
PERMANOVA main test and pairwise comparison results. df degrees of freedom, SS sum of squares.

| Source | df | SS | Pseudo-F | P |
|---|---|---|---|---|
| Zone | 5 | 1.6874E5 | 17.372 | 0.001 |
| Res | 451 | 8.7612E5 | | |
| Total | 456 | 1.0449E6 | | |
| **Pairwise-comparisons** | | | | |
| | | t | P | |
| Zone 1 vs Zone 2 | | 4.1189 | 0.001 | |
| Zone 1 vs Zone 3 | | 4.7309 | 0.001 | |
| Zone 1 vs Zone 4 | | 5.3913 | 0.001 | |
| Zone 1 vs Zone 5 | | 7.512 | 0.001 | |
| Zone 1 vs Zone 6 | | 6.6397 | 0.001 | |
| Zone 2 vs Zone 3 | | 1.2563 | 0.160 | |
| Zone 2 vs Zone 4 | | 2.7825 | 0.001 | |
| Zone 2 vs Zone 5 | | 5.5481 | 0.001 | |
| Zone 2 vs Zone 6 | | 4.5016 | 0.001 | |
| Zone 3 vs Zone 4 | | 2.5727 | 0.001 | |
| Zone 3 vs Zone 5 | | 5.4522 | 0.001 | |
| Zone 3 vs Zone 6 | | 4.3348 | 0.001 | |
| Zone 4 vs Zone 5 | | 3.5948 | 0.001 | |
| Zone 4 vs Zone 6 | | 3.1669 | 0.001 | |
| Zone 5 vs Zone 6 | | 1.6365 | 0.026 | |

**Table 5**
SIMPER analysis results for each zone; “Av.Abund” is the average value of the species abundance in the zone; “Av.Sim.” is the average for Bray-Curtis similarity values; “Contrib%” is the percentage contribution to similarity, cumulated in “Cum%” until the cut-off > 95 % is reached.

| Species | Av.Abund | Av.Sim | Contrib% | Cum.% |
|---|---|---|---|---|
| **Zone 1** | | | | |
| SG | 3.56 | 60.39 | 98.76 | 98.76 |
| **Zone 2** | | | | |
| SG | 1.46 | 16.06 | 49.20 | 49.20 |
| TM | 1.03 | 5.90 | 18.09 | 67.29 |
| BP | 1.25 | 5.49 | 16.83 | 84.12 |
| SS | 0.94 | 3.02 | 9.25 | 93.37 |
| PB | 0.70 | 1.23 | 3.76 | 97.14 |
| **Zone 3** | | | | |
| SG | 1.35 | 9.30 | 29.97 | 29.97 |
| TM | 1.22 | 8.35 | 26.91 | 56.88 |
| BP | 1.39 | 7.03 | 22.66 | 79.53 |
| SS | 1.02 | 3.07 | 9.90 | 89.43 |
| PB | 0.90 | 1.66 | 5.35 | 94.79 |
| PG | 0.72 | 0.93 | 3.00 | 97.78 |
| **Zone 4** | | | | |
| BP | 1.69 | 7.66 | 24.95 | 24.95 |
| SS | 1.68 | 6.68 | 21.75 | 46.70 |
| SG | 1.04 | 6.30 | 20.52 | 67.22 |
| PG | 1.50 | 3.56 | 11.59 | 78.82 |
| TM | 0.86 | 3.14 | 10.21 | 89.03 |
| PB | 1.28 | 2.15 | 7.01 | 96.04 |
| **Zone 5** | | | | |
| SS | 2.21 | 11.37 | 31.08 | 31.08 |
| BP | 1.63 | 6.53 | 17.87 | 48.95 |
| PB | 2.07 | 5.33 | 14.57 | 63.53 |
| AA | 1.64 | 4.12 | 11.27 | 74.79 |
| PG | 1.62 | 4.07 | 11.14 | 85.93 |
| CC | 1.22 | 2.40 | 6.58 | 92.51 |
| CB | 1.04 | 0.78 | 2.13 | 94.64 |
| RB | 0.67 | 0.73 | 2.00 | 96.63 |
| **Zone 6** | | | | |
| SS | 1.90 | 10.79 | 32.22 | 32.22 |
| CC | 1.80 | 8.42 | 25.14 | 57.36 |
| PG | 1.50 | 4.64 | 13.84 | 71.20 |
| PB | 1.37 | 3.44 | 10.28 | 81.48 |
| BP | 1.16 | 3.36 | 10.02 | 91.50 |
| AA | 0.67 | 1.71 | 5.11 | 96.61 |

respectively); in zone 4, the species contributing most was BP (25 %); in zones 5 and 6, the species contributing most was SS (31 % and 32 %, respectively). To verify that the new reference communities were representative for the study area, the frequencies of the 16 indigenous species among all 457 samples was calculated, showing that those not included in the new reference communities by the SIMPER analyses had low frequencies (Figure S2).

A general shift in the species composition from zone 1 to zone 6 was observed (Table 6). Zone 1 (mountain ecology) was associated with a high representation of the salmonid species SG. Intermediate zones (2, 3 and 4) were characterized by a progressive presence of rheophilic taxa (TM, BP, SS, PB, PG) and with a decreasing representation of SG. TM decreased from zone 3 to zone 4. Lowland zones (5 and 6) were associated with the presence of rheophilic-limnophilic (CB, RB) and limnophilic species (AA, CC). The new expected fish communities were defined for each zone according to the results of the SIMPER Analysis (Table 6).

### 3.2. Sub-metric $X_{2,b}$ species-specific refined reference condition

Table 7 shows the threshold values for the calculation of the NISECI sub-metric $X_{2,b}$, obtained with the Michaelis-Menten function for the estimated density (Figure S3).

### 3.3. General NISECI and refined NISECI application test

NISECI was applied using both the zones from GRCs as well as the resulting zones from the RRCs (Table S3; Fig. 2). Results of the general NISECI application showed no site within Class I (high ecological status), 16 of the sites (67 %) in Classes II and III (good and moderate ecological status), and eight sites (33 %) were in Classes IV and V (poor and bad ecological status; Table 8). The application of the NISECI index with RRCs showed a clear improvement in the judgments on the ecological status (Table 8, Fig. 3). Despite the absence of sites in Class I, the assignments of good ecological status increased to the detriment of classes IV and V. Within this application, 20 sites (83 %) were in Classes II and III, and 4 sites (17 %) were in Classes IV and V.

The values of the NISECI, quality classes and each metric changed between the two applications (Table 9). In 18 sites (75 %) the NISECI value increased with the RRC, while in six sites (25 %) it decreased. Quality class increased in nine sites (38 %) with the RRC and decreased in three sites (13 %), while in 12 sites (50 %) it remained the same. $X_1$ increased in 14 sites (58 %) with the RRC and decreased in 10 sites (42 %). $X_2$ increased in 12 sites (50 %) with the RRC, decreased in seven sites (29 %) and remained the same in five sites (21 %). $X_3$ increased in three sites (12.5 %) with the RRC, decreased in three sites (12.5 %) and remained the same in 18 sites (75 %).

### 3.4. RRC effects evaluation

Assessments carried out by the three experts (Fig. 4) showed two main differences compared to the application of NISECI with both reference conditions (Fig. 3). There was no site assigned as bad ecological status (Class V) and two experts out of three assigned the high ecological status (Class I) in 8 % and 4 % of cases, while in both NISECI applications this quality class was never observed.

Comparison among the three expert judgements returned no statistical significance (Kolmogorov-Smirnov test; $P > 0.05$). The means of their frequency values were compared with the results of the general NISECI and showed a relatively weak agreement (Kolmogorov-Smirnov test; $0.05 < P < 0.3$), while the comparison with the refined NISECI returned a much stronger agreement (Kolmogorov-Smirnov test; $P > 0.999$).

## 4. Discussion

Using the NISECI index as a biological evaluation tool in Italian rivers is mandatory for fish community classification and management integrated with the WFD (Macchio et al., 2017; Rossi et al., 2021). The

**Table 6**
Expected species in the six zones.

| Species (code) | Zone 1 | Zone 2 | Zone 3 | Zone 4 | Zone 5 | Zone 6 | Species ecology |
|---|---|---|---|---|---|---|---|
| *S. ghigii* **(SG)** | X | X | X | X | | | salmonid |
| *T. muticellus* **(TM)** | | X | X | X | | | rheophilic |
| *B. plebejus* **(BP)** | | X | X | X | X | X | |
| *S. squalus* **(SS)** | | X | X | X | X | X | |
| *P. bonelli* **(PB)** | | X | X | X | X | X | |
| *P. genei* **(PG)** | | | X | X | X | X | |
| *C. bilineata* **(CB)** | | | | | X | | rheophilic/limnophilic |
| *R. benacensis* **(RB)** | | | | | X | | |
| *A. alborella* **(AA)** | | | | | X | X | limnophilic |
| *C. carpio* **(CC)** | | | | | X | X | |

**Table 7**
Threshold values for NISECI sub-metric $X_{2,b}$ for each species involved in the study. Values corresponding to 1st and 2nd tertile calculated with the Michaelis-Menten function for the estimated density (number of estimated specimens per $m^2$). Since data were not enough to allow the calculation of specific threshold for each zone, the same values were applied to all zones.

| Reference code | Species | 1st tertile | 2nd tertile |
|---|---|---|---|
| **AA** | *Alburnus alborella* | 0.050 | 0.151 |
| **BP** | *Barbus plebejus* | 0.012 | 0.046 |
| **PG** | *Protochondrostoma genei* | 0.006 | 0.025 |
| **CB** | *Cobitis bilineata* | 0.004 | 0.019 |
| **CC** | *Cyprinus carpio* | 0.014 | 0.040 |
| **RB** | *Romanogobio benacensis* | 0.001 | 0.006 |
| **SS** | *Squalius squalus* | 0.066 | 0.186 |
| **TM** | *Telestes muticellus* | 0.024 | 0.091 |
| **PB** | *Padogobius bonelli* | 0.004 | 0.019 |
| **SG** | *Salmo ghigii* | 0.036 | 0.129 |

NISECI, however, shows limits and difficulties in its application, mostly related to the definition of the reference conditions and their selection of expected species (Pagani et al., 2021; Rossi et al., 2021). Since the NISECI expresses a judgment on the observed fish community by measuring its deviation from an optimal theoretical condition, establishing appropriate reference conditions is fundamental (Macchio et al., 2017; Rossi et al., 2021). The issue regarding inaccurate reference communities has been known since the development of NISECI (Rossi et al., 2017, 2021) and the need to refine the reference conditions (Zerunian et al., 2009; Macchio et al., 2017) in relation to the geographical and ecological context of the water body under consideration is consistent with Ministerial Decree (2010). Nevertheless, during the intercalibration exercise within the WFD it became clear that the refinement process may have been hindered by the lack of pristine habitat, in both Italy (Rossi et al., 2022) and other EU countries (Jepsen and Pont, 2007), which implies the need to use a modeling approach for the refinement. While an objective approach for *spatially based* refinement was proposed for the NISECI metric $X_{2,a}$ (Rossi et al., 2021), this study presented a method for the refinement of reference conditions for the metric $X_1$ (presence/absence of indigenous species) and for the sub-metric $X_{2,b}$ (demographic consistency of indigenous species) to be specifically applied within the Reno basin and the eastern basins (afferent to the Adriatic Sea) of Emilia-Romagna. Notwithstanding the limited regional applicability of the RRCs developed in the present study, the methodology can be replicated in other regions or sub-regions to refine the metrics throughout Italy.

Six homogeneous zones and their associated fish communities were identified. They were always dominated by one or a few fish species and showed clear separations between the environmental groups (Table 6, Table 7). Classifications of zones in relation to fish communities resulted as follows (Table 6): the zone with mountain characteristics was associated with salmonid species (*Salmo ghigii*) of torrential environments, while the zones with lowland characteristics were associated with rheophilic-limnophilic species (*Cobitis bilineata, Romanogobio benacensis*) and limnophilic species (*Alburnus alborella, Cyprinus carpio*). The presence of the most strictly rheophilic species (*Telestes muticellus*) is linked to the zones with intermediate characteristics between mountain and lowland environments. It is also notable that certain species which ascribe to rheophilic ecology (*Barbus plebejus, Squalius squalus, Padogobius bonelli, Protochondrostoma genei*) are not strictly related to specific zones, suggesting that these species can adapt to a wider spectrum of environmental variability. The species lists characterizing these communities were significantly reduced when compared to those indicated by Zerunian et al. (2009). Species with very rare frequency in the dataset, such as *A. anguilla*, *C. gobio* or *B. caninus*, were not associated with any zone and several species such as *Tinca tinca* (Linnaeus 1758) and *Esox lucius* (Linnaeus 1758) were historically diminished in the area and were not found even in the oldest samples dating back to 1995. Threshold values for sub-metric $X_{2,b}$ provided rather accurate reference values for the listed species and yielded reasonable results as testified by the expert judgment analysis. Zone 2, characterized by an over-representation of uncovered rock with wide altitudinal variability, is specific to the Eastern Emilia-Romagna region and is unlikely to occur in other geographical regions. Excluding zone 2, the new zones increased from three (Zeruinian et al. 2009) to five. However, available data were not sufficient to define different thresholds for the sub-metric $X_{2,b}$ in the different zones, so zones 3 and 4 are treated as the same zone in the practical application of NISECI (i.e. they share the same expected indigenous species list and $X_{2,b}$ thresholds). This limits the practical increase of zones and highlights the need for an increase of collected data for further refinement activities in this or other geographical areas. Moreover, the list of species and population density reference thresholds should only be applied to the specific investigated area, as both fish species and their densities are unlikely to be comparable even at the level of the northern Italian macro-region. The gain in obtaining more accurate reference conditions by increasing the number of zones and refining the expected autochthonous species list has the drawback of limiting the geographical area where RRCs can be applied, making it necessary to perform analogous refinement procedures in the other areas of Italy. Nevertheless, the methodology used has the advantage of being based on objective environmental characterization of the sites, rather than the broader and less objective definition of the classical three zones (Zerunian et al., 2009), and a similar approach is currently being used to define the same information for other regions. It could also be argued that the higher number of zones makes NISECI calculations more laborious, as the refinement procedure was complex and required the specific statistical analyses and elaborations presented in this study. However, once the RRCs are developed and provided to the regional environmental protection agencies (e.g., ARPAE), the calculation of NISECI by field sampling operators follows the standard NISECI protocol and would not require more laborious approaches by the technical personnel who routinely performs the field sampling.

The application of the refined NISECI resulted in a clear improvement of ecological statuses and a substantial shift towards the good ecological status (Class II). The greatest effects of refinement were

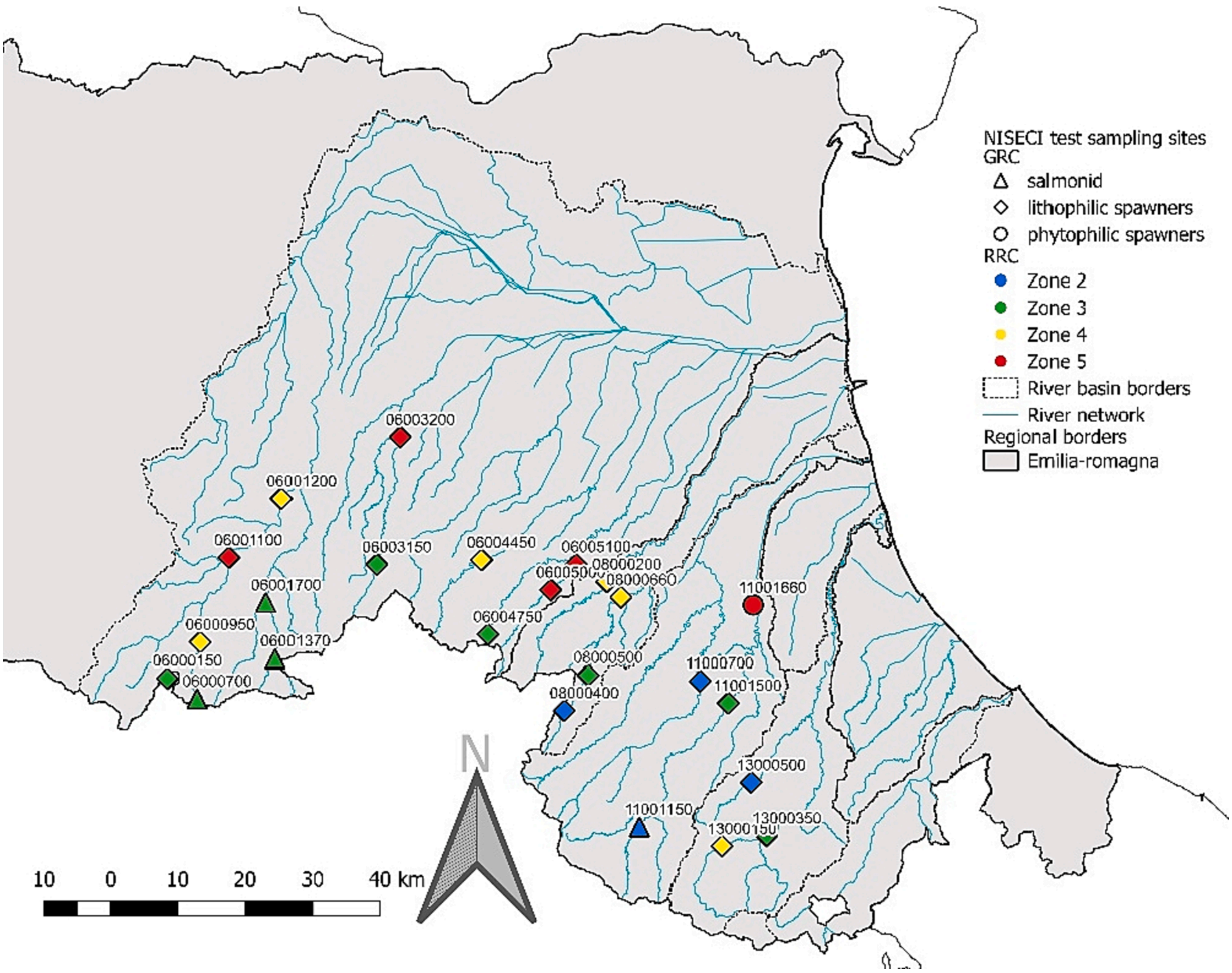


**Fig. 2.** NISECI test sampling sites distribution. For each sampling site, the ARPAE site ID is shown; GRC is indicated by the shape while RRC is indicated by the color.

**Table 8**
Comparison between GRCs (G) and RRCs (R) for each metric, for the NISECI value and for the corresponding quality class. The judgements of quality class by the three experts are also reported as E1-E3.

| Site ID | Site Name | X1 | | X2 | | X3 | | NISECI | | Quality Class | | E1 | E2 | E3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | G | R | G | R | G | R | G | R | G | R | | | |
| 1 | 06000150 | 0.444 | 0.846 | 0.325 | 0.260 | 1.000 | 1.000 | 0.239 | 0.319 | III | III | II | I | II |
| 2 | 06000700 | 1.000 | 0.385 | 0.300 | 0.100 | 0.675 | 0.000 | 0.382 | 0.112 | II | V | II | I | I |
| 3 | 06000950 | 0.556 | 0.615 | 0.340 | 0.425 | 0.875 | 0.875 | 0.280 | 0.348 | III | II | III | II | III |
| 4 | 06001100 | 0.556 | 0.375 | 0.380 | 0.467 | 0.925 | 0.925 | 0.303 | 0.270 | III | III | II | II | III |
| 5 | 06001200 | 0.667 | 0.769 | 0.267 | 0.320 | 1.000 | 1.000 | 0.276 | 0.341 | III | II | II | II | II |
| 6 | 06001370 | 1.000 | 0.385 | 0.000 | 0.350 | 0.675 | 0.675 | 0.097 | 0.221 | V | III | III | II | II |
| 7 | 06001700 | 0.000 | 0.615 | 0.000 | 0.525 | 0.000 | 0.925 | 0.000 | 0.406 | V | II | II | II | II |
| 8 | 06003150 | 0.333 | 0.462 | 0.267 | 0.267 | 0.925 | 0.925 | 0.179 | 0.216 | IV | III | II | II | II |
| 9 | 06003200 | 0.667 | 0.750 | 0.517 | 0.517 | 0.792 | 0.792 | 0.420 | 0.459 | II | II | III | III | III |
| 10 | 06004230 | 0.778 | 0.769 | 0.214 | 0.260 | 1.000 | 1.000 | 0.268 | 0.299 | III | III | II | II | II |
| 11 | 06004750 | 0.667 | 0.769 | 0.383 | 0.420 | 0.925 | 0.925 | 0.345 | 0.408 | II | II | II | II | III |
| 12 | 06005000 | 0.222 | 0.125 | 0.300 | 0.400 | 0.925 | 0.875 | 0.154 | 0.137 | IV | IV | III | II | III |
| 13 | 06005100 | 0.333 | 0.500 | 0.200 | 0.150 | 0.925 | 0.925 | 0.155 | 0.168 | IV | IV | III | III | IV |
| 14 | 08000200 | 0.778 | 0.615 | 0.386 | 0.525 | 0.875 | 0.875 | 0.385 | 0.404 | II | II | II | II | II |
| 15 | 08000400 | 0.333 | 0.308 | 0.500 | 0.550 | 0.925 | 0.875 | 0.260 | 0.262 | III | III | III | III | III |
| 16 | 08000500 | 0.333 | 0.545 | 0.433 | 0.433 | 0.925 | 0.925 | 0.237 | 0.326 | III | II | III | III | III |
| 17 | 08000660 | 0.556 | 0.462 | 0.760 | 0.700 | 0.875 | 0.875 | 0.493 | 0.405 | II | II | III | III | III |
| 18 | 11000700 | 0.667 | 0.545 | 0.583 | 0.567 | 0.675 | 0.675 | 0.454 | 0.384 | II | II | II | II | II |
| 19 | 11001150 | 0.600 | 0.818 | 0.000 | 0.250 | 0.875 | 0.925 | 0.076 | 0.302 | V | III | II | II | II |
| 20 | 11001500 | 0.444 | 0.308 | 0.350 | 0.300 | 0.925 | 0.925 | 0.248 | 0.183 | III | IV | II | II | II |
| 21 | 11001660 | 0.267 | 0.625 | 0.150 | 0.180 | 0.000 | 0.650 | 0.110 | 0.204 | V | III | IV | IV | IV |
| 22 | 13000150 | 0.556 | 1.000 | 0.540 | 0.450 | 0.792 | 0.792 | 0.380 | 0.516 | II | II | III | III | II |
| 23 | 13000500 | 0.333 | 0.545 | 0.267 | 0.267 | 1.000 | 1.000 | 0.180 | 0.242 | IV | III | III | II | III |
| 24 | 13000350 | 0.444 | 0.615 | 0.400 | 0.400 | 0.875 | 0.875 | 0.269 | 0.334 | III | II | IV | III | IV |

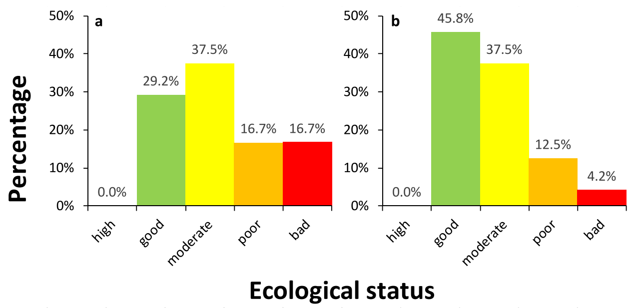


**Fig. 3. Frequency of the five ecological status judgements** with the general (a) and refined (b) NISECI assessment.

**Table 9**
Variation in results between general and refined NISECI application.

| | X1 | X2 | X3 | NISECI | Class |
|---|---|---|---|---|---|
| **Increased** | 58 % | 50 % | 12.5 % | 75 % | 38 % |
| **Decreased** | 42 % | 29 % | 12.5 % | 25 % | 13 % |
| **Equal** | 0 % | 21 % | 75 % | 0 % | 50 % |

observed for the $X_1$ metric and for NISECI values, which varied in all cases. A significant effect was observed for the $X_2$ metric which had a strong positive effect on the NISECI value, suggesting how crucial the reference community is for both metrics relating to the autochthonous components and subsequently, the final index result. The increased values are due to the reduction of the expected autochthonous species list in the RRCs and do not indicate a higher accuracy of the index, which was assessed with specific tests based on expert judgement.

The opinion of experts (i.e., someone having specialist knowledge acquired through practice, study or experience, e.g., Booker and McNamara, 2004; Kangas and Leskinen, 2005; O'Leary et al., 2009; Kuhnert et al., 2010) is widely used in ecology, as they may provide the best available information when measured data and formal theories are lacking in complex environmental systems (Krueger et al., 2012). Expert opinion helps to make the published and unpublished knowledge and the wisdom of experts explicit, while also providing a temporary summary of the limited available knowledge before conclusive scientific evidence becomes available (Kangas and Leskinen, 2005; Knol et al., 2010). The number and types of experts formally consulted in environmental modelling studies varies from one to 25 (Krueger et al., 2012). In this study, three experts were consulted, which can be considered sufficient as a representative sample (Clemen and Winkler, 1999; Krueger et al., 2012), especially given the regional context limited to Eastern Emilia-Romagna. Furthermore, gains in robustness may be too small to outweigh higher costs of additional expert recruitment (Clemen and Winkler, 1999; Knol et al., 2010). Expert uncertainty was not quantified here, but the expert-based judgments were remarkably consistent with each other, while their aggregated judgement showed clear mismatches with the GRC NISECI. The results from this study, however, show that the application of the RRC NISECI strongly agrees with the experts, confirming that the refinement method increased the analytical accuracy of the index.

The New European Fish Index (EFI+), based on the previous EFI index (Pont et al., 2006), complies with the WFD requirements and has been developed to propose its application across Europe (EFI+ Consortium, 2009). Considering that both EFI+ and NISECI refer to the WFD, they share similar aspects but are also very different in the metrics used. As we did in our study, also EFI+ developed a method to objectively classify river types based on abiotic variables and calibrated it across Europe using data from 15 countries (EFI+ Consortium, 2009). Considering the Italian territory, EFI+ defines two ecoregions, each one divided into the Salmonid and Cyprinid dominated river types (EFI+ Consortium, 2009), while NISECI considers 3 ecoregions, each one divided into the 3 river types Salmonid, lithophilic deposition Cyprinid, and phytophilic deposition Cyprinid (Macchio et al., 2017). The authors of NISECI point out that this zonation is too coarse, suggesting that the types should be further refined, as we did in our study. Another difference between the two indexes is that EFI+ metrics consider the functional role of the species, thus using the two broad groups of Salmonid and Cyprinid fish (EFI+ Consortium, 2009), while NISECI considers the representation and population health of autochthonous and hybrid/invasive species (Macchio et al., 2017). This makes it profoundly important to refine not only the river zonation for NISECI, but also the list of expected species that is specific to the local ecological history. The need for a greater level of detail in the reference conditions due to the high number of endemic species is reported for the Mediterranean ecoregion, such as the Balkan peninsula, Greece (Economou et al., 2007; Koutsikos et al., 2012; Koutrakis et al., 2013), and Italy (Bianco, 1995, 2014). The turkish-adapted version of EFI+ also provided a very narrow assessment range failing to accurately and consistently determine the severity of environmental degradation (Zogaris et al., 2023).

In conclusion, this study provided a method for the redefinition of reference fish communities through an objective approach, which is replicable in other geographical areas of Italy associated with available and sufficient databases. As next steps for the future direction and development of the NISECI index, it is necessary to address the following aspects: a) increasing data availability for rare or absent species, possibly with targeted samplings in water bodies with environmental peculiarities, especially in the plains areas which are poorly represented in the dataset and to which many of the missing species are linked; b) objectify the zone assignment for new sites by analyzing the most influential environmental variables in the classification and identification of threshold values.

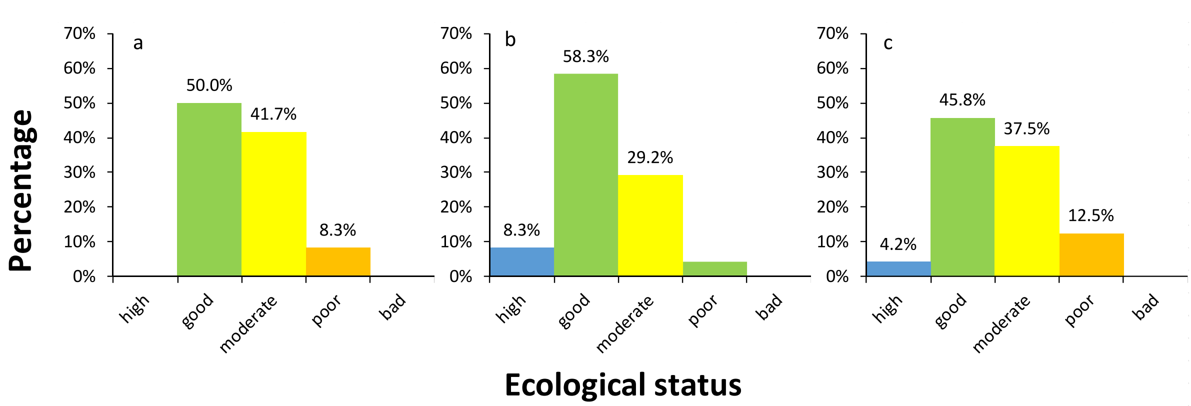


**Fig. 4.** Frequency of the ecological status judgement by the three experts (a-c).

## CRediT authorship contribution statement

**Andrea Marchi:** Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Visualization. **Andrea Bertaccini:** Formal analysis. **Wenqu Fan:** Formal analysis. **Gianluca Zuffi:** Investigation, Resources. **Stefano Sacchetti:** Investigation, Resources. **Matteo Nanetti:** Investigation, Resources. **Chloe Lee:** Writing – review & editing. **Alessandra Agostini:** Resources, Writing – review & editing. **Daniela Lucchini:** Resources, Supervision, Funding acquisition. **Silvia Bianconcini:** Methodology. **Francesco Zaccanti:** Methodology, Investigation, Resources. **Stefano Goffredo:** Resources, Writing – review & editing, Funding acquisition. **Erik Caroselli:** Conceptualization, Methodology, Resources, Writing – review & editing, Visualization, Supervision.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2023.111070.

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