WATER FRAMEWORK DIRECTIVE 3rd INTERCALIBRATION PHASE

MEDITERRANEAN GEOGRAPHICAL INTERCALIBRATION GROUP

COASTAL WATERS

BIOLOGICAL QUALITY ELEMENT PHYTOPLANKTON

CROATIA, ITALY AND SLOVENIA WORKING DOCUMENT

State of the affairs - NOVEMBER 2014

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Summary

Rome – 24 November 2014

1. Preamble

This document contains the results of the elaborations that led to the definition of the ecological classification criteria for the CW BQE Phytoplankton. Before moving on to illustrate these results we believe appropriate to set the point on a few basic concepts, with the purpose to facilitate the understanding of the work that has been done, especially in relation to the procedures adopted.

a) Concerning the way we followed to define the classification criteria, in particular to test the sensitivity of the chosen metrics (G_means and 90th p. of chlorophyll concentrations) to the gradient of the pressures and to set the boundaries among classes, we have adopted an empirical-statistical approach, quite similar to the approach was already adopted by OECD-Vollenweider for lakes (OECD-Vollenweider, 1982), to say:

   - Correlations between nutrient loadings, nutritional conditions in coastal areas (e.g. with respect to the N:P ratios variation) and trophic indicators (i.e. nitrogen and phosphorus concentrations at sea).
   - Regressions between biomass indicators (i.e. chlorophyll) and nutritional factors (nutrients concentrations), as pressure indicators.

   It is worth mentioning that coastal areas cannot be equated to the concept of the CSTR ("continuous stirred tank reactor") model, to say that cannot be considered as a confined system such as a single lake water body, with a "well defined" catchment area from where quantitatively "well defined" nutrient loads enter the lake. We decided therefore to use the "Dilution factor" of the seawater (F_dil: % content of freshwater in a sample of seawater), as a pressure indicator potentially related to nutrient inputs from the continent (Giovanardi and Tromellini, 1992b), as further explained, in a more detail, in the following. By the way, the F_dil approach was also used in a realistic way, to derive reference conditions for each category of coastal waters.

b) <u>Establishment of the boundaries.</u> Once we have identified the pressure factors that affect the variability of chlorophyll, it remains however the difficulty to determine the boundaries between classes, between what is acceptable and what is not. As it is well known, the WFD clearly defines how it should be expressed the judgment of ecological status and on what indicators must be based: to say, *inter alia*, specific composition and relative abundances of Phytoplankton. This approach has been for long time explored, with encouraging results also (Facca *et al*., 2014. France, 2009), but of little practical use for coastal waters, at least for the moment, given the difficulty of defining appropriate diversity indices and find significant functional relationships with the main pressure factors, and this regardless of the availability of suitable time series of Phytoplankton species composition.
   For these reasons, the judgment of the ecological status for the BQE Phytoplankton, i.e. the judgment on what is acceptable and on what is not (G/M boundary), has been referred to the scale of the water quality conditions described by the TRIX scale (Tab.1).

TRIX annual means	Trophic Status	Water quality Conditions
<4	Elevated (oligotrophy)	• Scarcely productive waters. • Good water transparency. • Absence of anomalous water colour. • Absence of Oxygen under-saturation conditions in the bottom waters.
4-5	Good (mesotrophy)	• Moderately productive waters. • Occasional water turbidity. • Occasional anomalous water colour. • Occasional bottom water hypoxia.
5-6	Mediocre (eutrophy)	• Very productive waters. • Low water transparency. • Frequent anomalous water colour. • hypoxic and occasional anoxic episodes in the bottom layers. • Some degradation of benthic communities.
>6	Bad (hypereutrophy)	• Strongly productive waters. • High water turbidity. • Diffuse and persistent anomalies in water colour. • Diffuse and persistent hypoxic/anoxic episodes in the bottom waters. • High mortality rate of benthic organisms. • Alteration of the benthic communities and strong decrease of the biodiversity

Tab 1 *Reference values for annual TRIX means, corresponding trophic status and related coastal water quality conditions.* (From Rinaldi and Giovanardi, 2011)

TRIX Index and the related scale were developed in the nineties of the past century (Vollenweider et al., 1998) for the eutrophied waters of Emilia Romagna coasts and it has been for long time used as a management tool. Prior to the receiving the WFD, this index was required by Italian Legislation on the protection of the coastal environment. TRIX has been also adopted by the UNEP MED POL against the eutrophication risk in the Mediterranean Region. In conclusion, we believe that the scale of quality conditions shown in the table is more than adequate to qualitatively describe what is represented indeed by a quantitative value of the average annual concentration of chlorophyll (or by the 90^{th} percentile of the related statistical distributions).

So, trying to summarize:

1) we use the Dilution factor as an indicator of “potential” contribution of nutrients from the continent (*per se*, the F_dil indicator does not represent a pressure parameter in the true sense of the word, but it is indisputable that the input of nutrients in a coastal area should be strictly related to the fresh waters of continental origin).

2) By means of the stepwise regression techniques, we have demonstrated that F_dil becomes often redundant with respect to the total phosphorus concentrations, in determining the variability of the chlorophyll in a coastal environment. Consequently, we have tuned the chlorophyll variation range depending on TP concentration values, starting from the datasets specially crafted for each type of coastal waters (Type II “Tyrrhenian”, Type I and Type II “Adriatic”). TP become therefore the principal indicator that define the pressure gradient and on which the sensitivity of the G_means of chlorophyll have been tested, as adequate metrics to assess the status for the BQE Phytoplankton in coastal waters.

3) About the use of TRIX Index. TRIX is a linear combination of the four fundamental trophic status indicators (chlorophyll, nitrogen, phosphorus and oxygen deviation from saturation). As such, TRIX represents a mixing of pressure (N and P) and impact (Chl-a and oxygen deviation) indicators and finally it does not result consistent with the WFD “philosophy”. But for our purposes, we are mainly interested to the trophic scale associated to the TRIX values, just because we believe that the quality conditions described by the scale are instead consistent with the WFD requests for the BQE Phytoplankton in coastal waters.
   It is all too clear that any attempt to find a relationship between TRIX and each of its components will be strongly affected by spurious correlation. On the other hand the contribution of each component to the final formulation of the TRIX is quite variable, especially if we compare the Tyrrhenian datasets (where the abiotic component -N and P- tends to prevail), with those related to the Adriatic sea (where instead prevail the weights of chlorophyll, as biomass indicator, and oxygen deviation from saturation, as intensity of production indicator) (Giovanardi and Vollenweider, 2004). Inevitably, if we want to assign to the concentration gradient of TP and chlorophyll the same descriptive scale for the water quality conditions as identified by TRIX, we have to put in a diagram TRIX values against TP and chlorophyll data. In other words, we have to use TRIX as a "control variable", just to arrange the corresponding Chl and TP boundary values that define the limits of the classification criterion we want to build up. In this way, we are also able to distinguish the different behavior of the two seas, the Tyrrhenian and the Adriatic, so peculiar in their trophic regimes, and to justify the need to assume two distinct typologies with their respective distinct classification criteria.

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- *Technical note about statistical properties of chlorophyll and nutrients data distributions.*

Regardless of the Types, we have considered chlorophyll *a* data distributions as functionally related to phenomena of multiplicative type, like biomass growth and nutrients uptake and release.

Therefore, we always reason in terms of annual geometric means (G_means, i.e. the arithmetic means of Chl log-data re-converted into numbers), since the statistical distributions of chlorophyll and nutrients tend to log-normality. Consider that log-transformation leads to normalize what, by its own nature, is of exponential kind. About these topics, a rich literature is available: e.g. Margalef, R., 1965; OECD-Vollenweider, 1982; Innamorati and Giovanardi, (1992); (Giovanardi and Vollenweider, 2004).

It follows that, having fixed the average, also the percentiles of the distributions are automatically defined, including the 90th percentile, i.e. the metric which they are based on the classification criteria adopted.

The 90th percentile has been therefore calculated as:

$$\text{Chl-a } 90^{th} \text{ p.} = 10\text{^}(\text{Log}_{10}\,(\text{G\_mean Chl-a}) + 1.28 \times \text{SD}).$$

By definition, the normalization of the chlorophyll distributions by means of log-transformation gives rise to the stabilization of the variances, with a standard deviation (SD), practically constant (around 0.3-0.4: Giovanardi and Tromellini, 1992a). Later in the following elaborations, we will use SD = 0.35 for Adriatic Type I, SD = 0.33 for Adriatic Type II and SD = 0.30 for Tyrrhenian Type II Log_{10}-transformed data, as it emerged from the analysis of long data series and related distributions of chlorophyll, used for the purposes of the intercalibration exercise.

2. Previous information

As agreed at the 3rd MED GIG Meeting in Rome (January, 2011), Croatia (HR), Italy (IT) and Slovenia (SI) used only biomass, specifically chlorophyll-a concentration (Chl-a) for the intercalibration exercise. Explanation and justifications could be found in Annexes 5.1 and 5.2 of the Milestone 5 report of the 2nd IC phase (Mediterranean Geographical Intercalibration Group, 2011).

No boundary setting was possible at the national level with Croatian and Slovenian data alone, mainly because of very similar trophic conditions which characterize the coastal waters of the East Adriatic coast. The three MSs (HR, IT and SI) agreed therefore to prepare a common methodological approach for the Adriatic Sea, which would rely on the comprehensive knowledge on the eutrophication problem of the area (Vollenweider et al., 1992; Cozzi et al., 2012; Mozetič et al., 2010).

During the 2nd IC phase, a common data set for all participating countries was built. The three MS used these data for the development of a common methodology as it represents a unique opportunity for such a task. All the details on the sampling and analytical compatibility could be found in the Milestone 5 report (MedGIG, 2011).

The common data set included 799 samples (89 stations/year) for the period 2007-2009. As no systematic data on land-based pressures were available at the level of the whole Adriatic basin, in a comparable manner, the total phosphorus (TP) concentrations in the seawater have been used as a proxy of pressures. It was found after a stepwise regression analysis on the common data set, that the impact (the dependent variable, in terms of Chl-a concentration) resulted well explained by the Dilution factor (F_dil) and by TP content, as regressors. In any case the results showed that TP accounts for the maximum weight in determining the variability of chlorophyll, for both types (Type I and Type II Adriatic); the other regressors, although significant, have lowest effects (see Milestone 5 report: par. ad 4.2., page 45). These results indicate that the phosphorus pool in the water column is an internal measure of the phosphorus enrichment from outside. In this regard, we draw your attention to the fact that in its most part, the Adriatic Sea is a phosphorus-limited sea (Chiaudani and Vighi 1982; Maestrini et al. 1997; Pojed and Kveder 1977).

For the Adriatic Sea, the most of the data used in the previous analyses represent type I and IIA waters, following the criteria agreed in the 1st IC phase (MedGIG, 2007).

As for the Tyrrhenian Sea, some attempts have been made to intercalibrate Type II waters with FR an SP, but, mainly due to the very different responses to “Land Uses Simplified Index” (LUSI) by the Tyrrhenian coastal systems, not comparable to the results obtained with the LUSI for Type II coastal waters of FR and SP, it was agreed to stop trying intercalibration exercise with FR and SP (Milestone 5 report of the 2nd IC phase; MedGIG, 2011) and to consider Type II

A “Tyrrhenian” as a Type of its own. That decision was definitively confirmed by the entire WG CW Phytoplankton, at the meeting held in Barcelona (March 2014).

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So, this was the state of the art and the progress of work at the end of the second phase of the intercalibration exercise for CW BQE Phytoplankton. The results of this phase have been published on the COMMISSION DECISION of 20 September 2013 “establishing, pursuant to Directive 2000/60/EC of the European Parliament and of the Council, the values of the Member State monitoring system classifications as a result of the intercalibration exercise and repealing Decision 2008/915/EC”. The Classification criteria defined by the CW Phytoplankton Working Group were however included in the Annex II. As it is known, *“Annex II to this Decision sets out the results of the intercalibration exercise for which intercalibration is partially achieved. The completion of all the necessary steps in the intercalibration exercise should be carried out in order for the results to be included in a new Decision. Accordingly, those results are provisional.”*

The major criticisms that were raised were mainly concerned with the procedural aspects, to say, in short:

- Different approaches in determining pressures indicators and fixing boundary setting.
- Very questionable procedure adopted to derive Reference Conditions, (with Chl-a ref. values not differentiated between Adriatic and Tyrrhenian Types II).
- Excessive resulting gap between the values assigned to the Chl-a Ref. Cond. compared with those proposed by SP and FR. The same applies to the boundaries defined for Type II “Tyrrhenian”, too low if compared to those proposed by FR and SP for the same type of water category.

We believe we have provided sufficient justification and clarification in the large preamble of this document. In the following, we describe in detail the revised procedures and the results obtained in each step. This has led to even substantial changes with respect to the results provided at the end of the II IC Phase, in particular for the case of Type II “Tyrrhenian” and for the RC values assigned to the Types.

In any case the:

- a) Evaluation of the Reference Conditions,
- b) Pressures Impact Relationships
- c) Boundary setting and Classification Criterion definition,

will be treated <u>separately</u> and analysed for each of the related Types: Type I “Adriatic”; Type II A “Adriatic”; Type II A “Tyrrhenian” and Type III W for both Adriatic and Tyrrhenian Seas.

3. Reference conditions evaluation

WFD CIS Guidance Document No. 5 establishes that reference conditions (RC) represent the values of the biological quality elements that exist, or would exist, in a certain water body type at high ecological status, i.e. water body type with no, or very minor disturbance from human activities. The objective of setting reference condition standards is to enable the assessment of ecological quality against these standards.

As already mentioned above, the Dilution factor[1] has been utilised to derive RC, since we have assigned to this parameter the meaning of comprehensive pressure indicator, specifically related to the potential transport of nutrients (natural loads plus anthropogenic loads), from the mainland to the sea. As it can be easily understood, to equal contributions of freshwater from the continent, the amount of nutrients associated to these inputs can differ from area to area, in relation to many factors (land use, degree of waste water treatment, etc.). This is the reason why the weight of the F_dil indicator, as a regressor against Chl-a values, is generally lower than the much larger phosphorus weight in determining the Chl-a variability.

Then you add the dynamics of the currents at a local level, as e.g. in the case of the Northern Adriatic, where the mixing of the freshwaters from the Po river source with the open seawaters, can be strongly affected by prevailing current direction, vertical and horizontal advection, eddy formations, wind stress, tidal cycles, etc. The quantification of these effects would be too complex, but it is unimportant for our purposes, since F_dil can always be calculated from simple measurements of salinity, used as a tracer.

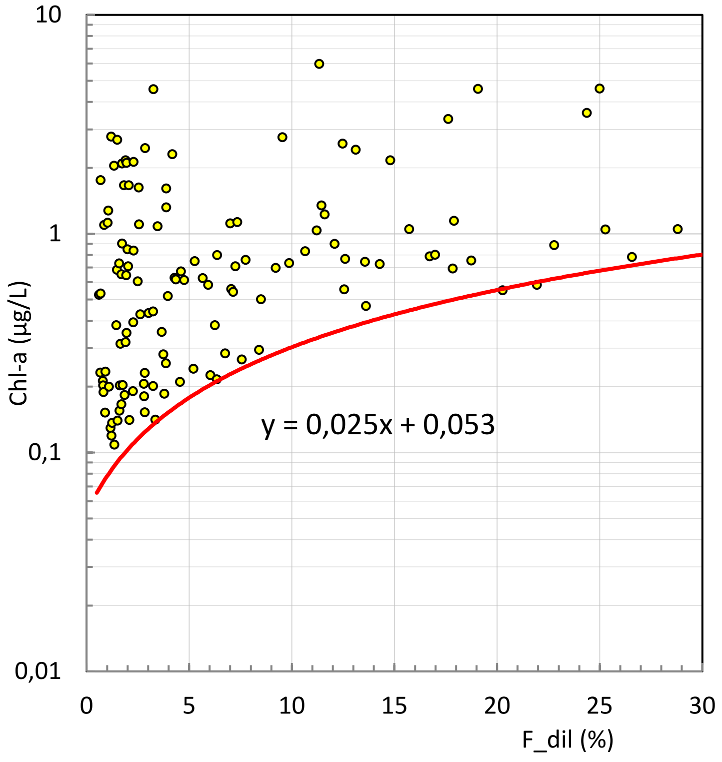


Fig. 1 *A scatter plot of annual geometric means of Chl-*a *of the common Mediterranean dataset plotted against the Dilution factor (F_dil). We interpret the red line as a measure of the natural trophic levels of coastal areas, not yet impacted by anthropogenic loads of some importance.*

[1] The Dilution factor (F_dil), defines the freshwater content of the sea (Yentsch 1975). It is calculated as: *F% =[(S-s)/S]*100*, where S = open sea salinity; s = measured salinity.

The diagram reported in Fig.1 had already been presented in the Milestone 5 report. It is repeated here because it is well suited to understand the meaning we assign to the Chl-a Reference Conditions. The data points are referred to the entire coastal development of the Mediterranean MSs (except Greece and Malta), in order to ensure maximum of the variation range. In this case, we are not interested in finding some significant relationship between Chl-a and F_dil, there is no regression line plotted in the graph. Instead, we have drawn a boundary-line between two different regions of the graph: there are no data-points below this red line. For each fixed value of the F_dil indicator, corresponding Chl-a values can range from a maximum of 6-7 μg/L (as annual G_means) to a minimum identified by the separation line, that we interpreted as the threshold between natural and anthropogenic pressure. We assume that the nutrient loads, presumably those originated naturally, or those generated by some human activities with however minor impact, determine a response from the coastal systems well represented by concentrations of Chl-a lying on the separation curve shown in the chart. Thus, the assessment of reference conditions for BQE Phytoplankton does not derive from theoretical considerations or expert judgments, but it refers to actual situations such as those occurring along the Mediterranean coasts, as evidenced by the long series of data provided by the monitoring activities carried out by the MSs.

A final consideration concerns the variation of the RC Chl-a concentrations as a function of the Dilution factor. In this case, the best relationship resulted of a linear kind (the equation reported in the diagram represents a straight line), and allowed to evaluate changes in the RC value as a continuous variable, functionally related to a wide spectrum of salinity values. For single Adriatic and Tyrrhenian Types considered separately, the best found relationships between RC Chl-a and F_dil resulted instead of exponential kind.

3.1. Type I

For this Adriatic Type, referred only to the eutrophied coastal belt of the Emilia Romagna Region (Italy), an area directly affected by Po River inputs, we have integrated the data belonging to the original common dataset (2007-2009) with new data related to the period 2002-2006 (Data base Sidimar[2]). The sampling stations considered were 14, from on-shore to 3000 m off-shore. In order to keep under control the inter-annual variability, several annual means were calculated for each sampling station, for a total of 68 data-points (Fig. 2).

[2] The Sidimar database makes available the data of coastal monitoring programs carried out, from 2000 to 2009, by the 15 Italian maritime regions in agreement with the Italian Ministry for the Environment (Directorate for Nature Protection -DPN)

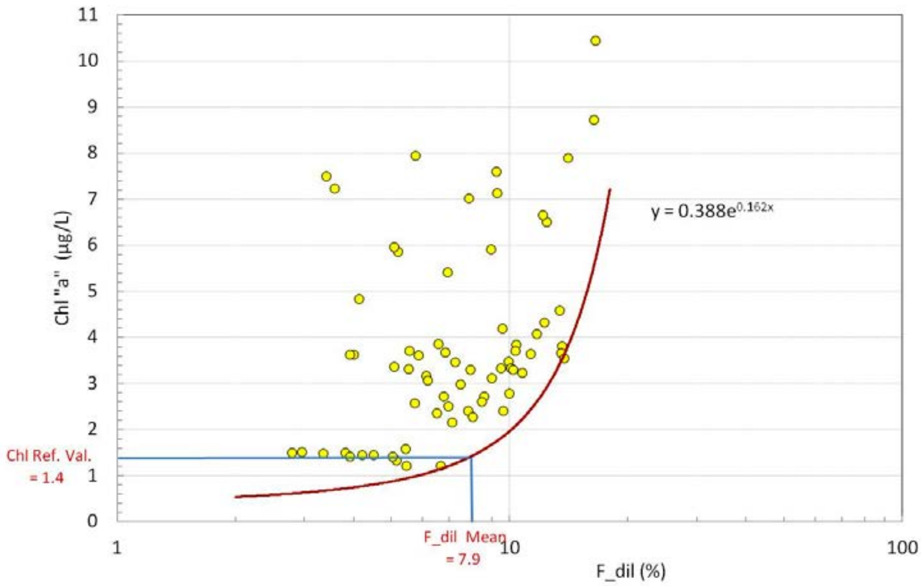


Fig. 2 *Type I "Adriatic" - Reference conditions for chlorophyll. Annual G_means of Chl-a data are plotted against the Dilution factor range of variation.*

The derived RC value for Chl-a has been then calculated from the corresponding value of F_dil = 7.9 (i.e. the overall average of the Dilution values, calculated for the entire period and for the whole area). The value found (RC = 1.4 µg / L as Chl-a) is close to the average chlorophyll values relating to the sampling station N. 319, located 3 km off-shore of the Cattolica transect (90 km south of the Po river delta), already considered as a reference station by the Emilia Romagna Region (ARPA-EMR).

3.2. Type II A "Adriatic"

From the common database (HR-SL-IT, period 2007-2009), 37 sampling stations were chosen, belonging to Type II and included in the belt of a nautical mile from the coast. Italian sampling stations are related to three coastal areas of the Northern and Central Adriatic Sea and belonging to Veneto, Marche and Abruzzi (excluding therefore the Emilia Romagna Region already considered as Type I). (Fig. 3)

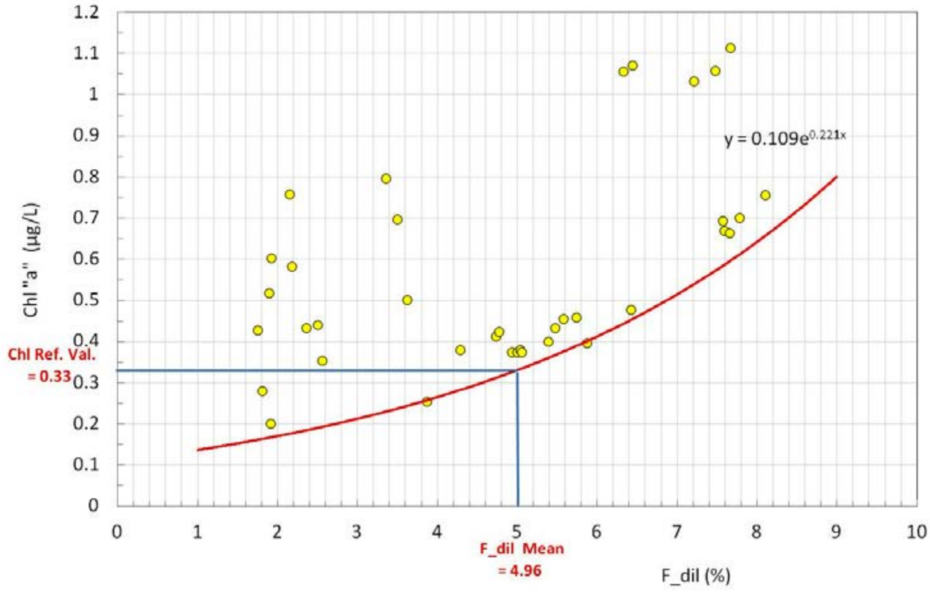


Fig. 3 *Type II "Adriatic" - Reference conditions for chlorophyll. Annual G_means of Chl-a data are plotted against the Dilution factor range of variation.*

The derived RC value for Chl-a has been calculated from the corresponding value of F_dil = 4.96 (i.e. the overall average of the Dilution values, calculated for the entire period on the whole common Type II dataset). The found RC value (= 0.33 µg/L as Chl-a), results very close to the Chl-a values characterizing on average Type III coastal waters (see later in the following).

3.3. Type II A "Tyrrhenian"

The old dataset already utilised in the second IC phase, has been integrated with new data from the rich Sidimar database. We have now processed 40 sampling stations along the Tyrrhenian and Ligurian coasts, referred to the period from 2002 to 2009 (Fig. 4).

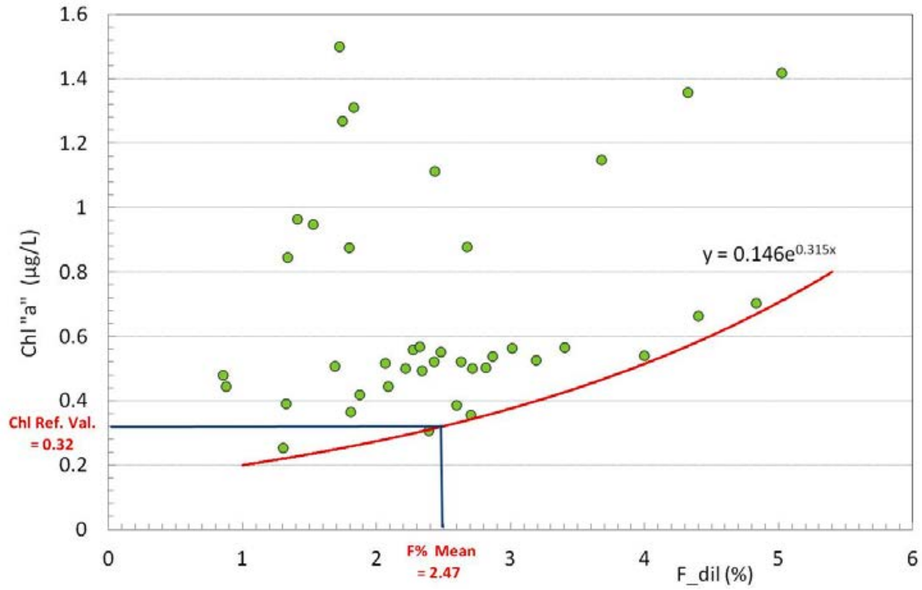


Fig. 4 *Type II "Tyrrhenian" - Reference conditions for chlorophyll. Annual G_means of Chl-a data are plotted against the Dilution factor range of variation.*

By means of relationships of exponential type, the derived RC value for Chl-a has been calculated from the corresponding value of F_dil = 2.47 (i.e. the overall average of the Dilution values, calculated for the entire period on the whole Type II “Tyrrhenian” dataset). The found RC value (= 0.32 µg/L as Chl-a), practically is the same already assigned to the Type II “Adriatic”. We can conclude that, for both seas and for Type II waters, the true natural conditions which may be taken as a "reference" for the BQE Phytoplankton (according to the above mentioned definition provided by the WFD Directive), are those approaching average conditions characterizing Type III coastal areas.

3.4. Summary table for BQE Phytoplankton reference conditions

	Type I	Type II-A ADRIATIC	Type II-A TYRRHENIAN
RC - G_Mean Chl-a, µg/L	1.4	0.33	0,32
RC - 90^{th} percentile Chl-a, µg/L	3.9	0.87	0.77

Note. The 90^{th} percentile value has been reported to make however possible the EQR calculations and for comparison with other methodologies and/or other Mediterranean areas. It is a theoretical value, based on the assumption of normality of the distributions of chlorophyll, after log-transformation of the raw data, as largely discussed above.

4. Pressure-Impact relationships

In this section, we provide the evidence of what has been broadly anticipated, on the evaluation procedures adopted about the relationship between pressures and impacts.

- *Adriatic Sea*

We repeat here, in Fig. 5, the same graph already presented in milestone 5 (TP against F_dil), although with some adjustment and modification (removal of a pair of Croatian sampling stations with F_dil values too low to belong to the Type II and elimination of some outliers).

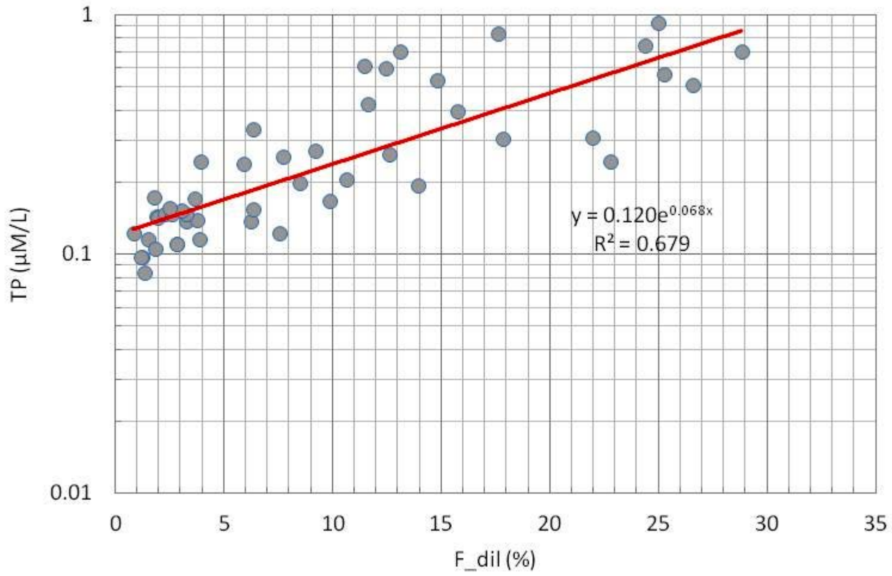


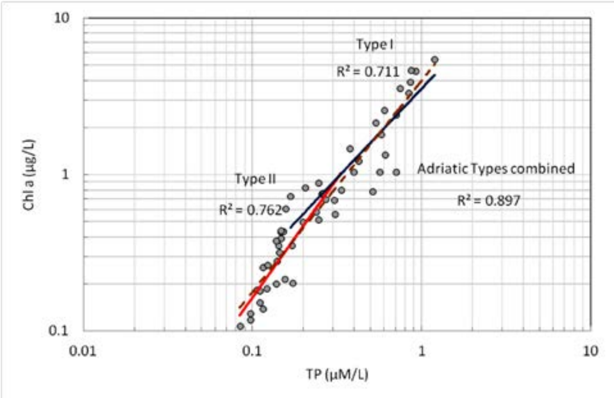
Fig. 5 *Adriatic Common dataset: functional relationships between TP (annual G_means) and Dilution Factor – Type I and Type II Sampling Stations combined.*

The graph is well suitable to make us understand that the relation shown represents a statistical *continuum*, it remains valid for waters of both Type I and Type II, irrespective of the level of pressure, which can be high or low depending on the values assumed by the Dilution factor. This implies that the TP concentrations observed at sea are closely and functionally to be related to the freshwater inputs from the continent, and then to be referred to the amount of nutrient loads generated and delivered from the basins burdening on the coastal areas.

Obviously the same functional relationship can be highlighted to the Dissolved Inorganic Nitrogen, with a significance level even higher (R^2 = 0.88), being the DIN a substance more conservative and soluble, if compared to TP. [3]

Based on these results, we have the right to say that the concentrations of nutrients in the sea represent true pressure indicators and, as such, can be used to test the pressure-impact relationship, using chlorophyll as an indicator that documents the response of coastal systems to nutrient availability.

[3] The phosphorus associated to the freshwater inputs from the continent is a less "conservative" substance than inorganic nitrogen, since its decay in seawaters must be referred not only to physical dilution, but also to the removal from the system due to sedimentation and/or chemical precipitation (Giovanardi and Tromellini, 1992).



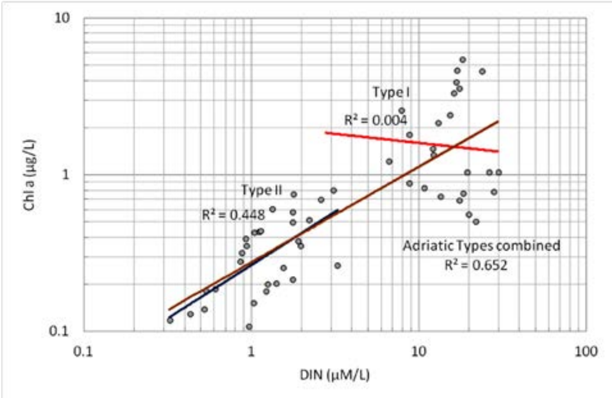


Fig.6 *Adriatic Common dataset: Chl-a (annual G_means) plotted against the TP and DIN.*

By plotting therefore the annual G_means of Chl-a against the corresponding gradients of TP and DIN concentrations respectively, we obtain the graphical representations as presented in Fig. 6.

From the inspection of the two diagrams, we can draw the following conclusions:

- chlorophyll, as principal indicator of phytoplankton biomass, is strongly related to the TP variations, considering the sampling stations, both separated and combined, belonging to Type I and to Type II. In any case, the resulting correlations are always highly significant. We can say that, along the coasts of the Northern and Central Adriatic Sea, the 90% of the overall Chl-a variability is explained by phosphorus that therefore assumes the role of “principal pressure factor” in relation to BQE Phytoplankton.
- Conversely, analysing the behaviour of the inorganic nitrogen, we can see that as pressure factor, it provides weak and non-significant correlations towards chlorophyll (Type II), or none at all (Type I). This confirms what has been widely reported in the literature (see e.g. the References already cited above), to say that, due the high N:P ratios in the Adriatic areas (>50), nitrogen does not limit the algal growth (i.e. nitrogen excess is not taken by Phytoplankton) and this leads to low or zero correlation values that we have found.

- ***Tyrrhenian Sea***

We have already had occasion to mention the differences in the trophic regimes that differentiate the two seas. In general, Tyrrhenian coastal systems react less efficiently to the availability of nutrients and tend to produce less biomass (in terms of concentrations of chlorophyll). Unlike the Adriatic, where the P-limitation represents almost a generalized condition, the Tyrrhenian coastal environments present conditions of phosphorus limitation mostly in correspondence of riverine inputs. We can say that the main effect attributable to the contribution of freshwater inputs from rivers, is represented by an increase in the ratio N:P, which therefore becomes itself a very good indicator of pressure, although the maximum values are unlikely to exceed N:P = 30, (cfr. Fig. 7).

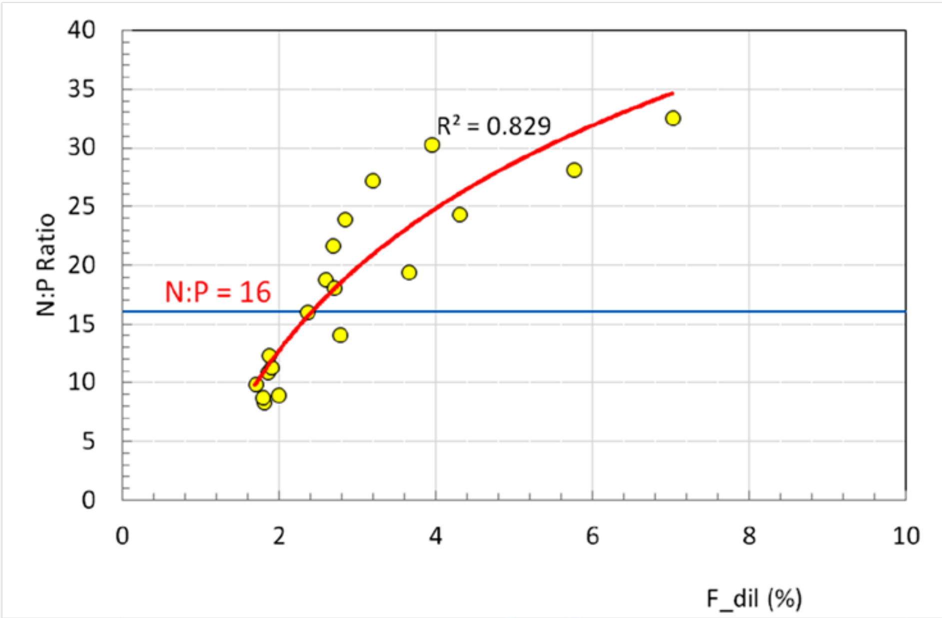


Fig. 7 *Tyrrhenian dataset (Type II A): N:P ratios variations (as annual G_means) against the F_dil gradient.*

Due to the high sensitivity of the N:P ratios towards the gradient of the F_dil parameter, we can guess that the value of the ratio is dominated by the variability of the numerator (i.e. DIN), rather than by the denominator (i.e. P-PO_4).

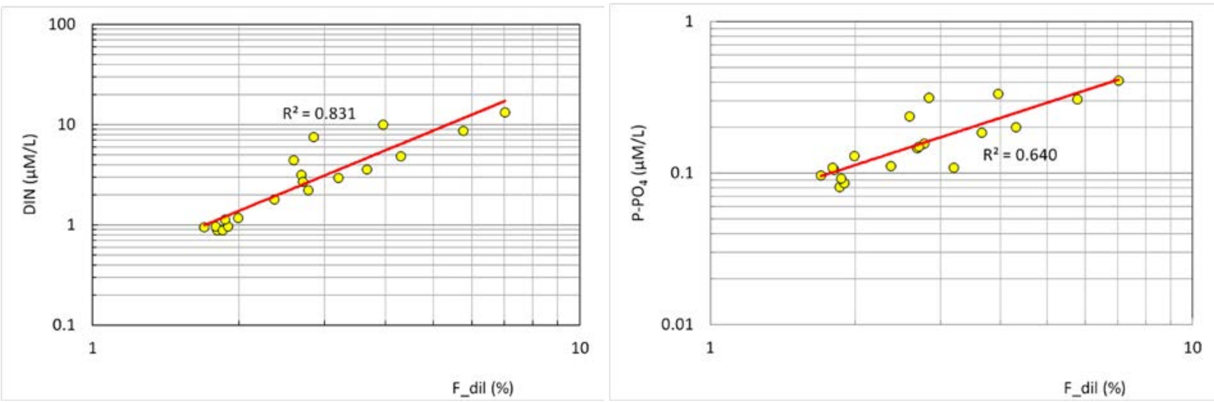


Fig. 8 *Tyrrhenian dataset: functional relationships between annual G_means of dissolved nutrients (inorganic nitrogen and ortho-phosphate), and Dilution factor.*

The diagrams presented in Fig. 8, shows (in the left panel) highly correlated variations of nitrogen vs the Dilution factor, with an increase from one to 10 micromoles/L of DIN, corresponding to a rise of 5 percentage points of the F_dil parameter.

Unlike nitrogen, the functional relationships between P-PO_4 and F_dil (see the right panel), are much weaker, although they resulted nevertheless statistically significant. Note that in the same variation range of the dilution, the orthophosphate concentrations increase only from 0.1 to 0.4 micromoles/L.

By replacing the orthophosphate with its total form (i.e. TP), we obtain a very similar relationship *versus* F_dil, with a level of even weaker correlation (R^2 = 0.62), though still significant.

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In the next sections, we address the issues of the pressure-impact relationships, to say the functional relationships between biomass indicators (i.e. chlorophyll), and nutritional factors (nutrients concentrations), as pressure indicators, using the same procedures for each Type separately.

Particular attention will be paid with respect to the type of relation found and to its statistical significance. We must remember that these relationships have the main purpose to test the sensitivity of chlorophyll to the gradient of the pressures. They also have the purpose to define the ranges of variation and, consequently, to fix the boundaries between the different classes of the classification criterion that we want to build.

This approach has involved the use of techniques of regression analysis. In this regard, we resorted to the useful statistical packages provided by the program R (R Development Core Team., 2005), with the related diagnostic tests, that ensure us not only on the significance of the functional link found, but also on its validity (verification of the normality of the residuals, the absence of autocorrelation between them, the probabilities associated to the regression coefficients, etc.). Obviously, all this information is available, if requested.

4.1. Type I

Concerning the Emilia Romagna coastal waters belonging to this type, we have processed the data related to 14 sampling stations, located in the belt of 3 km from the shore. The relevant period extends from 2002 to 2009, having integrated the original common dataset with new data provided by the database Sidimar, as mentioned above. This is therefore the reference period, the base line, to which the classification criterion based on chlorophyll must be reported.

For this water category, we have already shown that the pressure-impact relationships between inorganic nitrogen and chlorophyll produce a very low and not significant correlation. Therefore, we only consider the relationship Chl-a *vs* TP (Fig.9).

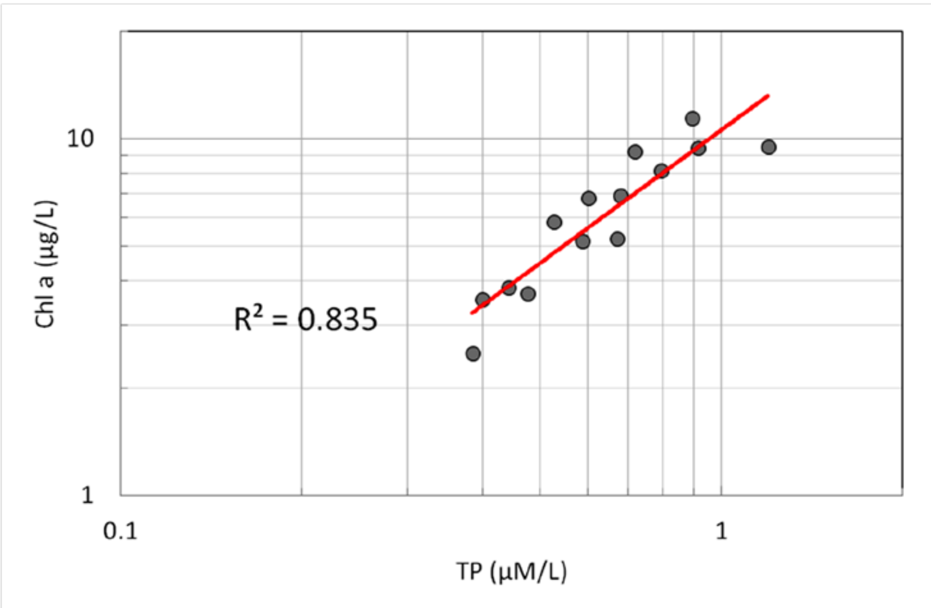

Fig. 9 Type I: regression between annual G_means of chlorophyll vs TP.

The best resulting relation is of a *log-log* type. Between numbers it becomes:

$$[\text{Chl-a}] = 10.591\ [\text{TP}]^{1.237} \qquad (4.1.1),$$

where:

- [Chl-a] are G_means of chlorophyll expressed as µg/L
- [TP] are the total phosphorus concentrations defining the pressure gradient (as µM/L)

This regression equation will allow us to derive the Chl-a class boundaries directly from the corresponding TP values, appropriately calibrated on the trophic scale identified by TRIX Index.

The following graph shows the resulting diagram that makes it possible to refer single TP concentrations to the corresponding TRIX values and to the related trophic scale.

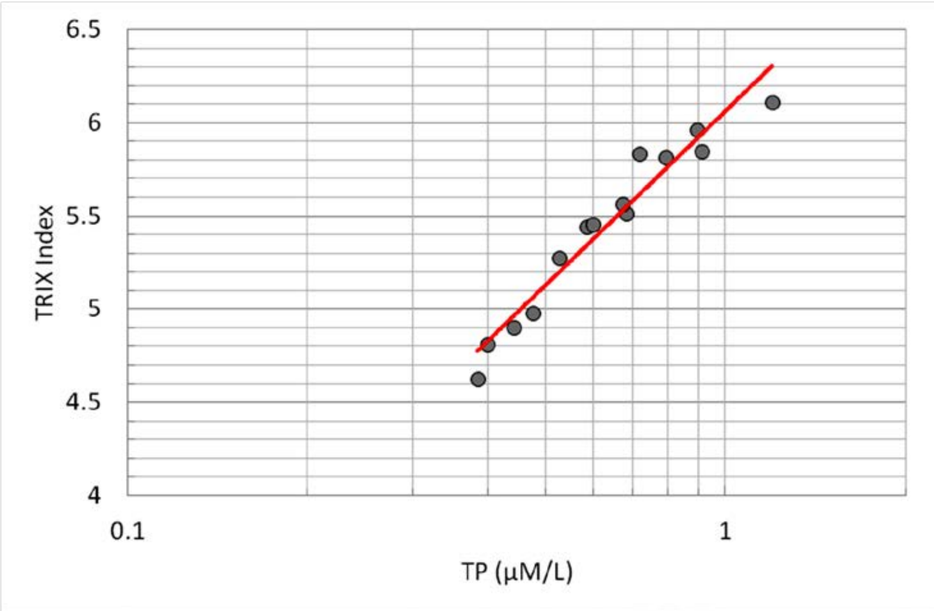

Fig. 10 *Type I - Relationship between total phosphorus and the related TRIX scale.*

The equation of the red line is of a *log*-type:

$$\text{TRIX value} = 1.349\ ln\ [\text{TP}] + 6.064 \quad (4.1.2.),$$

where *ln* [TP] stands for the natural logarithm of the TP concentrations, expressed as µM/L.

In order to derive single TP concentrations, it is necessary to solve the inverse of the equation 4.1.2.:

$$[\text{TP}] = exp\ [(\text{TRIX val.} - 6.064)/1.349] \quad (4.1.3.).$$

The Type I class boundaries of the Classification Criterion for BQE Phytoplankton will be therefore derived as follows:

- from Eq. 4.1.3., the limits of the class for TP concentrations, as pressure values corresponding to the boundaries of the TRIX trophic scale;
- from Eq.4.1.1., the Chl-a concentration limits, corresponding instead to the boundaries of the pressure TP values, just above calculated.

By the way, [Chl-a] and [TP] appearing in the equations, are to be meant as the annual G_means of the related parameters.

4.2. Type II A “Adriatic”

It is of a type common to the three Members States (HR, IT, SL). The pressure-impact relationships have been tested on the Common Data Base already utilized in the II IC Phase. The relevant period extends from 2007 to 2009, to be intended therefore as the reference period on which the classification criterion based on chlorophyll, will be tuned.

As for the case of Type I, we only consider the relation Chl-a *vs* TP, since the pressure-impact relationships between inorganic nitrogen and chlorophyll produce a very low and not significant correlation. We refers therefore to the graph already discussed above (Fig. 6), and we choose as the best relationship, the regression equation referred to the Adriatic Types combined, which produced the highest correlation (r = 0.95).

This equation is of a *log-log* type. Between numbers it becomes:

$$[\text{Chl-a}] = 3.978\ [\text{TP}]^{1.347} \qquad (4.2.1.)$$

where:

- [Chl-a] are G_means of chlorophyll expressed as µg/L.
- [TP] is the total phosphorus concentrations defining the pressure gradient (as µM/L).

The following graph reports the diagram used to derive TP concentration boundaries from the corresponding TRIX boundaries in the related trophic scale.

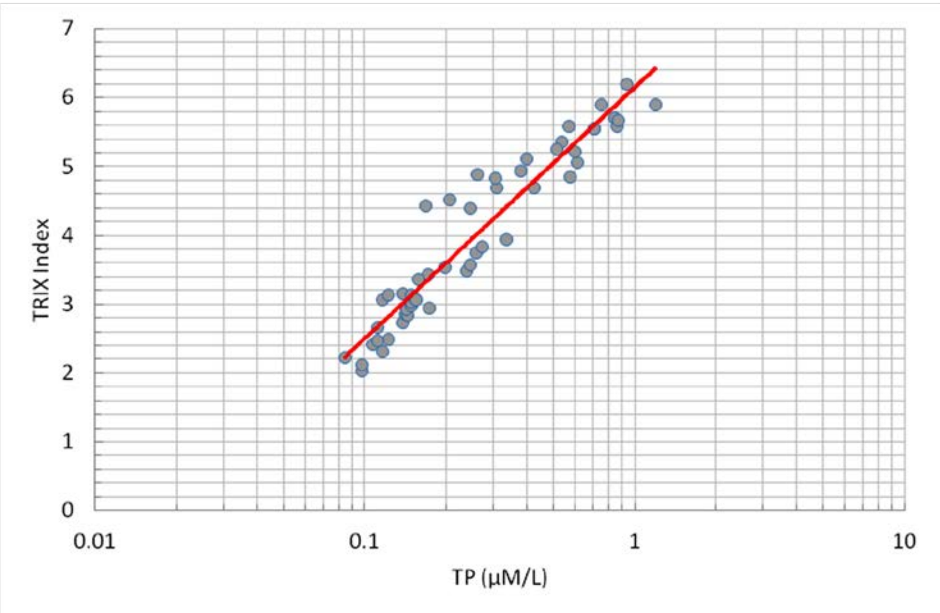

Fig. 11 Adriatic Common dataset: - Relationship between total phosphorus and the related TRIX scale.

The equation of the red line is of a *log*-type:

$$\text{TRIX value} = 1.583\ \mathit{ln}\ [\text{TP}] + 6.148 \qquad (4.2.2.),$$

As already said, *ln* [TP] stands for the natural logarithm of the TP concentrations, expressed as µM/L.

Single TP concentrations corresponding to a prefixed TRIX value, can be calculated from the inverse of the equation 4.2.2.:

$$[TP] = exp\ [(TRIX\ val. - 6.148)/1.583] \quad (4.2.3.).$$

The Type II A "Adriatic" class boundaries of the Classification Criterion for BQE Phytoplankton will be therefore derived as follows:

- from Eq. 4.2.3., the limits of the class for TP concentrations, as pressure values corresponding to the boundaries of the TRIX trophic scale;
- from Eq.4.2.1., the Chl-a concentration limits, corresponding instead to the boundaries of the pressure TP values, just above calculated.

4.3. Type II A "Tyrrhenian"

In order to evaluate the pressure-impact relationships for this typology of waters, the original Tyrrhenian dataset, already processed to derive Reference Conditions (see Section 3.3.), has been reduced to 19 sampling stations that actually belong to Type II, corresponding mainly to coastal areas directly affected by river mouths of some importance (Tiber, Arno, Ombrone), but also by minor rivers which however result in a relevant seasonal variability in salinities, at least at a local scale. The reference period is 2002-2009, to which therefore the classification criterion based on chlorophyll must be reported.

In these coastal areas, the phosphorus-limiting conditions tend to prevail over the natural conditions typical of the Tyrrhenian Sea, which is otherwise characterized by nitrogen-limitation.

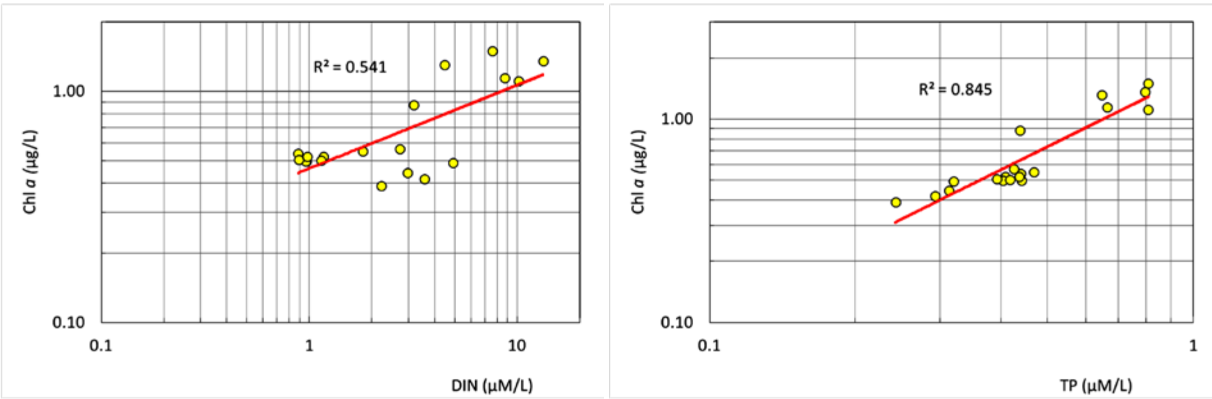

Fig. 12 *Type II A "Tyrrhenian" – Regressions between annual G_means of chlorophyll vs DIN and TP.*

Examining the diagrams in Fig. 12, we find a low degree of correlation between the main indicator of phytoplankton biomass, the chlorophyll, and corresponding inorganic nitrogen concentrations at sea. About the reasons for this weak functional link, we have already discussed above, talking about the role of the ratio N:P and its variations in coastal waters.

In the right panel of Fig. 12, the correlation between TP and Chl-a results instead highly significant. The regression line plotted in the graph is of a *log-log* type. Between numbers it becomes:

$$[Chl\text{-}a] = 1.656\ [TP]^{1.178} \quad (4.3.1.)$$

where, as usual:

- [Chl-a] are G_means of chlorophyll expressed as µg/L.
- [TP] is the total phosphorus concentrations defining the pressure gradient (as µM/L).

The TP concentration limits corresponding to TRIX index boundaries in the related trophic scale, can be obtained from the next diagram (Fig. 13).

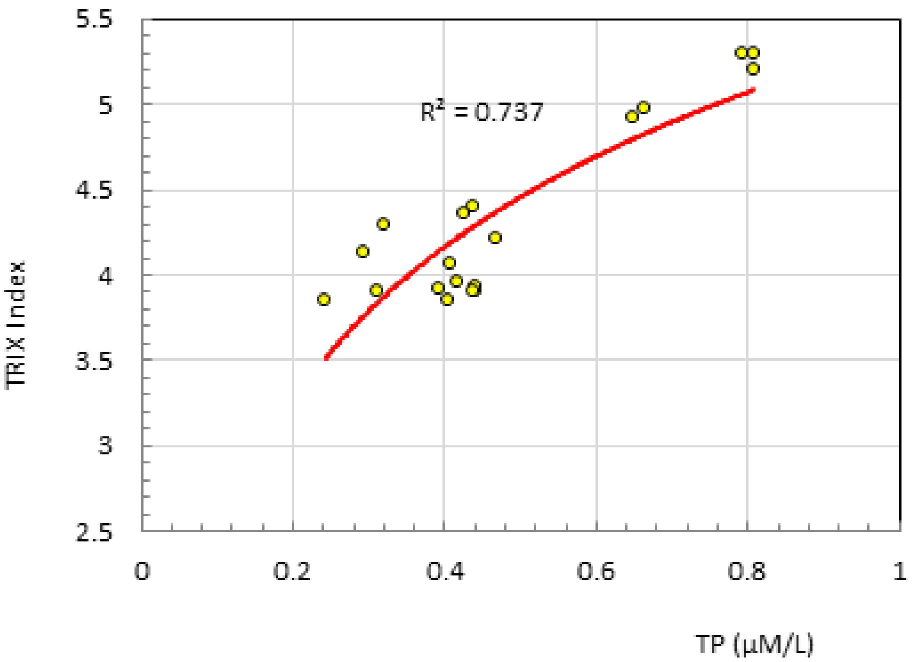

Fig. 13 *Type II A "Tyrrhenian" - Relationship between total phosphorus and the related TRIX scale.*

Although the points are not well aligned along the curve, due to the high variability of these Tyrrhenian coastal systems, we use the equation of this line to calculate:

$$\text{TRIX value} = 1.305\ ln\ [\text{TP}] + 5.363 \qquad (4.3.2.),$$

The inverse of the Eq. 4.3.2.:

$$[\text{TP}] = exp\ [(\text{TRIX val.} - 5.363)/1.305] \qquad (4.3.3.).$$

allows us to derive TP concentration values corresponding to a prefixed TRIX value.

Therefore, summarizing, Type II A "Tyrrhenian" class boundaries of the Classification Criterion for BQE Phytoplankton will be built as follows:

- from Eq. 4.3.3., the limits of the class for TP concentrations, as pressure values corresponding to the boundaries of the TRIX trophic scale;
- from Eq.4.3.1., the Chl-a concentration limits, corresponding instead to the boundaries of the pressure TP values, just above calculated.

5. Type III W

For this type of water category, common to both Tyrrhenian and Adriatic Sea coastal areas, we had already shown that, for classification purposes, chlorophyll was not a suitable metric, due to the difficulty to reach an acceptable discrimination level between two G_means of chlorophyll. At this regard, we refer to the WFD Intercalibration Phase 2: Milestone 5 report (see page 44), where these issues are discussed in detail. It was demonstrated that, with an yearly monitoring programme and a monthly sampling frequency, we would reach a discrimination level between two contiguous $\log_{10}$(Chl-a) averages equal to |0.25|, not indeed favourable for a status classification, when the range between two class boundaries could be fixed around ca. 0.24. With a weekly sampling frequency, the limit descends to 0.12, a value however, that does not yet help to provide an acceptable level of uncertainty.

It arises now a more significant impediment, related to the practical inability to find functional relationships between the gradient of the pressure and the measured values of chlorophyll.

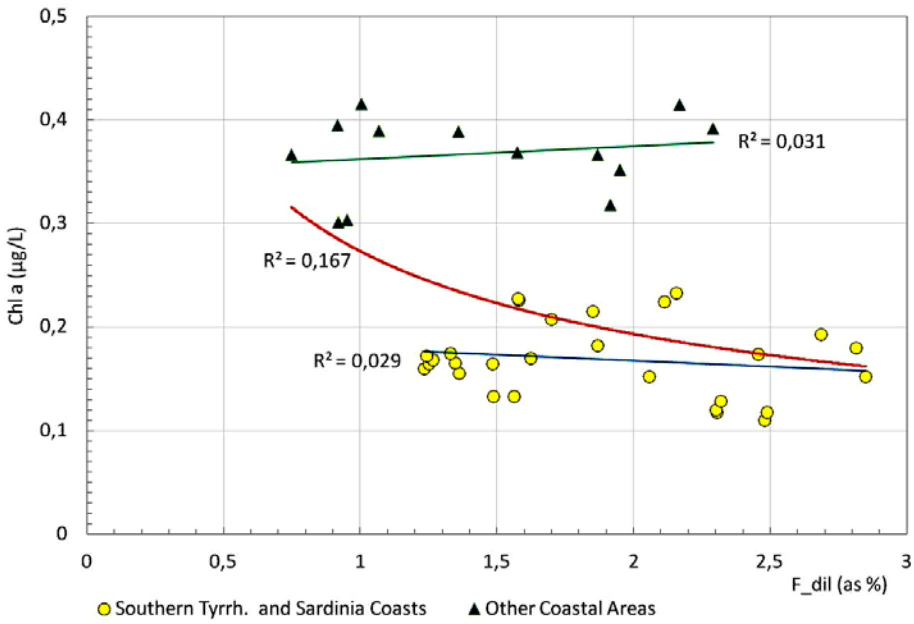

Fig. 14 Type III W – Chl a vs Dilution factor as a Presssure indicator.

Fig. 14 describes an attempt to find some connection between the annual G_means of chlorophyll per sampling station and the corresponding values of the Dilution factor. The data are referred to 41 sampling stations of the Southern Tyrrhenian Sea (Campania, Sicily and Sardinia coasts), Northern Tyrrhenian and Ligurian Sea (Tuscany) and Adriatic Sea (Croatian coasts).

It follows the complete lack of correlation between the two variables. The same applies if we replace the Chl-a G_mean concentrations with the corresponding nitrogen and phosphorus annual G_means: the correlations with the F_dil parameter are always not significant.

The distribution of the data points seems rather to reflect the general trophic characteristics that differentiate the strictly oligotrophic waters of the Southern Tyrrhenian Sea (Chl means < 0.3 µg/L), less productive than the Ligurian and the Northern Tyrrhenian surface waters (as well than the Adriatic Type III waters), with Chl-a values not exceeding however 0.4 µg/L, as annual G_mean.

As mentioned above, another distinctive trophic variable characterizing coastal environments, depending on the freshwater inputs coming from rivers and/or urban settlements, is represented by the N:P ratio. This ratio has been tested against the dilution factor (Fig. 15), since the N:P increase results always well correlated to the decrease of salinities, as clearly shown, according to the evidence, in the previous sections.

The chart in Fig. 15 allows us to make the following considerations:

- The Type III W coastal areas referred to the Tyrrhenian and Ligurian Seas are always characterised by N-limitation, with minimum N:P values around 8 in the sampling stations belonging to the Southern Tyrrhenian Sea. In any case these N:P values are well comparable with the data reported by the literature for the Surface Tyrrhenian Waters (Innamorati and Giovanardi, 1992), and appear to be typical of coastal areas not yet impacted by nutrient inputs of some importance.
  <u>We can therefore assume that, in the case of Tyrrhenian coastal waters, the transition from conditions of nitrogen-limitation to the phosphorous-limitation also marks the transition from Type III to Type II water category.</u>
- Conversely, the high N:P ratios characterizing Adriatic Type III W coastal areas, even if the values of chlorophyll recorded are very low, confirm the influence of the Po river, which tends to affect the whole Adriatic basin, in determining P-limitation conditions in the long (both spatial and temporal) terms, also in strictly oligotrophic areas (Cfr. the above cited bibliography on the Adriatic Sea).

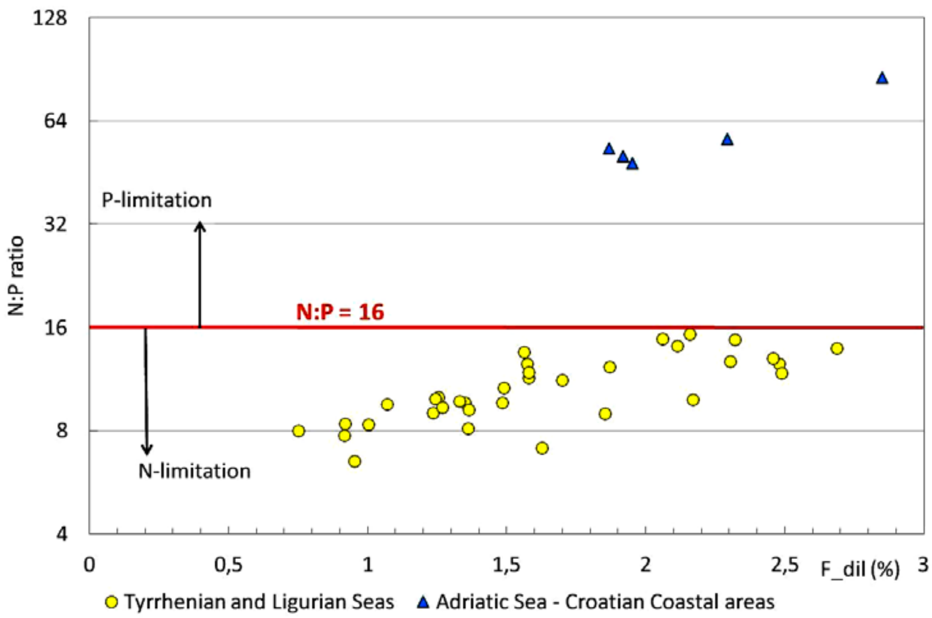

Fig. 15 *Type III W - N:P ratios* vs *Dilution factor*

6. CW BQE Phytoplankton: classification criterion and boundary setting

In the previous sections (Section 3:“Reference Conditions”; Section 4: “Pressure-Impact Relationships”), we have provided all the necessary tools to build up a Classification criterion for BQE Phytoplankton.

This criterion is based on the Chl-a values, as impact indicator, functionally related to a TP scale, as pressure gradient. The empirical relationships, obtained by means of the regression analysis, have been calculated from specific datasets, expressly prepared for each Type of water category.

As for the various Types, only Type II A “Adriatic” has involved the preparation of a common dataset among three MSs (HR, IT, SL). In this respect, since the three MSs have used the same database, adopted the same metric (Chl-a) and accepted common methodologies, there was no need to intercalibrate anything, nor even to harmonize the class limits.

For each Type, the G/M boundary has been set on the corresponding limit between Good and Mediocre Trophic Status, as defined by the TRIX scale (TRIX = 5; see the Table 1, reported in the preamble to this document). Using a more traditional terminology, TRIX = 5, marks the transition from meso-trophic to eu-trophic conditions. This boundary has been maintained both for Type II “Adriatic” and Type II “Tyrrhenian”. For Type I, it was decided to raise it by half a point (G/M boundary corresponding to TRIX = 5.5), to take account of nutrient loads coming from natural sources, that the river Po indeed conveys into the Adriatic Sea, presumably in not negligible amounts.

As for Type III W waters, we propose a threshold approach, by setting a limit of Chl-a annual G_means corresponding to the H/G boundary of the related Types II A “Adriatic” and “Tyrrhenian” respectively. This threshold value could assume the same meaning and the same constraints that the WFD assigns the G/M boundaries set for all other typologies, but this solution is questionable, since for Type III waters was not found any functional relationship with the gradient of the pressures.

One last comment concerns the division into classes, as stated by the WFD, and more precisely the P/B boundary. This boundary refers to a "virtual" condition, since from the data in our possession, was not possible to detect situations related to the class "Bad". TP concentration data characterizing this B class, have been extrapolated from functional relationships extended to an area of the diagrams not actually covered by observations and experimental data: we do not know how coastal systems behave with so high concentrations of phosphorus, especially since we are talking about annual averages. We therefore consider this class as indicative, but not strictly necessary for a proper ecological classification of the BQE Phytoplankton, based on chlorophyll.

Finally, in order to calculate exactly the EQRs values, normalized with respect to the actual values, the conversion functions for each Type, are reported in Fig. 16.

The boundaries for all the types are shown in the following tables.

Tab. 2 **Type I**

Boundaries	TRIX	Chl-a annual G_Mean (µg/L)	Chl-a 90th percentile (µg/L)	TP annual G_Mean (µmol/L)	EQRs actual	EQRs normalized
Ref. Value	-	1.40	3.93	-	1	1
H/G	4.5	2.5	7.1	0.31	0.56	0.83
G/M	5.5	6.3	17.7	0.66	0.22	0.61
M/P	6.5	15.8	44.3	1.38	0.09	0.40
P/B	7.5	39.5	110.9	2.90	0.04	0.19

Tab. 3 **Type II A “Adriatic”**

Boundaries	TRIX	Chl-a annual G_Mean (µg/L)	Chl-a 90th percentile (µg/L)	TP annual G_Mean (µmol/L)	EQRs actual	EQRs normalized
Ref. Values	-	0.33	0.87	-	1	1
H/G	4	0.64	1.7	0.26	0.52	0.82
G/M	5	1.5	4	0.48	0.22	0.61
M/P	6	3.5	9.3	0.91	0.09	0.40
P/B	7	8.2	21.7	1.71	0.04	0.19

Tab. 4 **Type II A “Tyrrhenian”**

Boundaries	TRIX	Chl a annual G_Mean (µg/L)	Chl a 90th percentile (µg/L)	TP annual G_Mean (µmol/L)	EQRs actual	EQRs normalized
Ref. Values	-	0.32	0.77	-	1	1
H/G	4	0.48	1.17	0.35	0.66	0.84
G/M	5	1.2	2.9	0.76	0.27	0.62
M/P	6	2.9	7.1	1.63	0.11	0.40
P/B	7	7.3	17.6	3.51	0.04	0.18

Tab 5 **Summary Table**

Type		Type I		Type II-A ADRIATIC		Type II-A TYRRHENIAN	
		G_Mean	90th p.	G_Mean	90th p.	G_Mean	90th p.
Ref. Conditions (Chl-a, µg/L)		1.4	3.93	0.33	0.8	0.32	0.77
Boundaries (Chl-a, µg/L)	H/G	2.5	7.1	0.64	1.7	0.48	1.17
	G/M	6.3	17.7	1.5	4.0	1.2	2.90
Boundaries (EQR normalized)	H/G	0.83		0.81		0.84	
	G/M	0.61		0.60		0.62	

Tab 6 **Type III W Chl-a Treshold values**

Types	Chl-a (µg/L) G_Mean	Chl-a (µg/L) 90th p.
Type III W “Adriatic”	0.64	1.7
Type III W “Tyrrhenian”	0.48	1.17

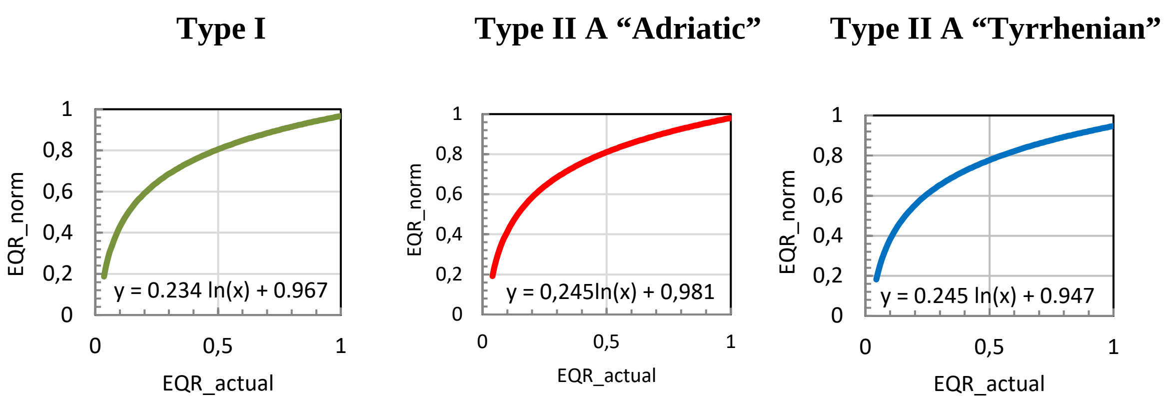

Fig. 16 Conversion functions for EQRs by Type.

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