Chapter 1

A SAMPLING PROTOCOL TO ASSESS THE ECOLOGICAL STATUS OF STREAMS AND RIVERS IN THE SPANISH MEDITERRANEAN AREA

INTRODUCTION

The Water Framework Directive (WFD) (European Parliament and Council, 2000) requires that the European countries need to assess the ecological status of their freshwater ecosystems using biological indicators (e.g. macroinvertebrates, fishes, macrophytes, riparian vegetation). Before 2016, the EU countries have to show to the Commission that their rivers and lakes are in a very good ecological status. In the United States, concepts as ecological health or biological integrity have been a key element for the water quality management and are included in environmental laws (Karr & Chu, 2000). As a consequence, there are several standardized methodologies to its assessment (Plafkin *et al.*, 1989; Barbour *et al.*, 1999; Carter & Resh, 2001). Similarly, in the last few years, some European countries have developed methodologies to assess the ecological status (Bloch, 1999; Chovanec *et al.*, 2000; Harper *et al.*, 2000). In Spain, despite the high number of studies about (and using) biological indices to establish river water quality (e.g., Alba-Tercedor *et al.*, 1992), there is not a standard methodology to be applied to water management.

To asses the river health status, different countries use Rapid Bioassessment Protocols (RBPs) (Wright *et al.*, 1984; Plafkin *et al.*, 1989; Davies, 1994; Tiller & Metzeling, 1998; Chutter, 1998; Barbour *et al.*, 1999). These methods are based in the evaluation of the biological integrity

(Karr, 1981, 1996) using habitat and biological quality assessment and a further comparison with the reference conditions (Barbour *et al.*, 1999; Resh *et al.*, 1995; Reynoldson *et al.*, 1997). The RBPs have been designed to be efficient, effective, easy to use, and low in cost and to be applied in wide regions (Resh & Jackson, 1993; Resh *et al.*, 1995). All this properties derive in a simplified sampling and processing of the samples, avoiding as much as can be possible, and the loss of information (Resh *et al.*, 1995; Barbour & Gerritsen, 1996).

One way for simplifying and optimizing the sampling is decreasing the number of samples per site (Resh *et al.*, 1995; Hewlett, 2000), integrating all the communities from the different habitats (Stribling *et al.*, 1993; Resh *et al.*, 1995), or sampling the "most productive habitat" present (Plafkin *et al.*, 1989). This approach has statistical implications because the lack of replicates for a site eliminates several parametric statistical methods from being used in analysis (Hulbert, 1984; Norris, 1995). However, the use of reference sites as replicates could avoid this problem (Norris, 1995; Resh *et al.*, 1995).

The processing of samples is a key factor in the use of RBPs designed for macroinvertebrates. Tiller & Metzeling (1998) and Metzeling & Miller (2001) proposed to sample and process the sample in the field during 30 minutes until 200 individuals were obtained. Other methods are time independent and the samples are processed in the lab counting 200 individuals, after taking the largest animals (Plafkin *et al.*, 1989; Barbour *et al.*, 1999). Barbour & Gerritsen (1996) showed that using a fixed number of individuals, the distribution patterns are similar from the ones using all the individuals. However, this subsampling procedure based in a fixed number of individuals could have implications in assessing the water quality, because the frequency and abundance of the rare taxa are affected (Cao & Williams, 1999; Cao *et al.*, 2001) and because it means that the organisms should have a homogeneous distribution (Countermanch, 1996). Due to that, other authors prefer a subsampling based in a fixed fraction (Cuffney *et al.*, 1993; Vinson & Hawking, 1996; Countermanch, 1996).

The rivers in mediterranean areas are subjected to high natural flow variability that implies the temporality of most of the rivers and streams and allows the presence of seasonally different macroinvertebrate communities (e.g., Gasith & Resh, 1999). Moreover, the human impacts are large: waste, flow regulation, riparian alteration, habitat alteration… (Prat, 1994; Prat & Ward, 1994; Prat & Munné, 2000). Consequently, methodologies developed in other countries are not directly applicable in these environments. The GUADALMED Project (see Limnetica, in press for a detailed description) is a Spanish funded project (HID98-0323-C05)

that attempts to assess the ecological status of the Mediterranean rivers and to establish the main factors implied on them. Six research teams belonging to different institutions in the country are implied: University of Barcelona, University of Vigo, University of Illes Balears, University of Murcia, University of Almeria, University of Granada and CEDEX. The main objective of the first stage of the GUADALMED Project was to establish, test and intercalibrate a standardized sampling Rapid Bioassessment Protocol to be adopted by the administration managers when the WFD is applied. The validation of the protocol is done under GUADALMED project for all the main watersheds draining into the Mediterranean sea in the Spanish coast (12 basins, 157 sites). In this paper we present data on the intercalibration exercise using macroinvertebrates.

The selection of a protocol to be used is based on the experience of the researchers of the project in the Iberian mediterranean rivers. Thus, for the biological quality establishment, two methodologies have been tested: IBMWP (Alba-Tercedor & Sánchez-Ortega, 1988; Alba-Tercedor, 1996; Alba-Tecedor & Pujante, 2000) and FBILL (Prat *et al.*, 1999); these two indices were chosen because they have been largely used in the area and proved to be sensitive to water quality. To evaluate the riparian vegetation status, the index QBR is used (Munné *et al.*, 1998; Suárez-Alonso & Vidal-Abarca, 2000; Munné *et al.*, in press).

METHODOLOGY

Study area

To select and intercalibrate the sampling and sorting methodology to be used in the project, we chosed a sampling site in the headwaters of the Argos stream (Barranda, Murcia), tributary of the Segura river (Figure 1). Argos stream is an intermittent stream with 48 km length, a slope of 18,6% and a drainage basin of 506 km^2. In the selected sampling site, the stream order is 4 and the altitude is 780 m. It's a site with low eutrophication, with hyposaline, alkaline waters, well oxygenated and hard and neutral waters (Table 1, from Vidal-Abarca, 1985). The channel substrate is mostly made by gravel, although sand, cobbles and bedrock can be found. Algae are abundant and dominated by Oscillatoriales, Nostocales, Cladophorales, Charales and Zygnematales (Aboal, 1988, 1989). The sampling was carried out in February 1999, which usually is close to the end of the wet season in this Mediterranean climate area.

Figure 1. Segura basin and sampling site in the Argos river.

Table 1. Physical and chemical parameters of Argos riverin the sampling area (from Vidal-Abarca, 1985).

	Mean values (Vidal-Abarca, 1985)		**Mean values** (Vidal-Abarca, 1985)
pH	8.6	Magnesium (mg/l)	70.3
Salinity (g/l)	0.52	Suspended Solids(mg/l)	46.5
Conductivity (µS/cm)	1203	Nitrates (µg/l)	50.7
Alkalinity (meq/l)	6.9	Nitrites (µg/l)	1.4
Chloride (mg/l)	100.1	Amonium (µg/l)	4.7
O_2 (mg/l)	11.1	Phosphates (µg/l)	2.2
Hardness (°F)	41.6	Silicates (µg/l)	161.8
Calcium (mg/l)	50.6	Chl-a (mg/l)	7.5

Sampling procedure

Working groups and site replication

The main goal of the study was to harmonize and homogenize the field methodology, especially the sampling and sorting of macroinvertebrates, between researchers of six different centers. All of them have large experience on macroinvertebrate studies. As the researchers of each center have differences in sampling and sorting, and some of them were not used to apply the QBR, we design the field experiment dividing the researchers in working teams. To avoid the individual effect the exercise was designed as follows:

1. The researchers from the six centers were divided into 4 teams. Each one had to sample, sort and count the macroinvertebrates from a site in the river Argos. Teams were composed by 4-5 people.
2. At least one member of each center was present in each group.
3. The sampling was made in four different sites of Argos stream 200 m away from each other. Care was taken in selecting the sites to avoid the differences of fauna due to different substrata composition.
4. The following protocol for sampling, determining the physico-chemical parameters, assessing the riparian vegetation and sorting the samples was establish previous to be applied in the exercise.

Macroinvertebrates

In each stream reach, two samples were collected from the riffles (R) and pools (L) habitats, using the kicking method. All the macroinvertebrates retained by a net of 250 μm mesh size were collected.

Two protocols to be compared were established, based on the one designed by Prat *et al.* (2000):

PROTOCOL 1: The samples were processed and identified in the field, except the most difficult taxa that were kept in alcohol 70% to be identified in the lab. In the field, the contents of the nets were put in plastic trays and the different taxa found were recorded and quantified in four ranks: 1 (1-3 indv.), 2 (4-10 indv.), 3 (10-100 indv.) or 4 (>100 indv.). This procedure would stop when after successive sorting no more new taxa appeared (Alba-Tercedor, 1996; Alba-Tercedor & Pujante, 2000).

PROTOCOL 2: The samples collected with the Protocol 1 were kept in alcohol 70% and were sorted and identified in the lab using a stereoscope. All the taxa that were seen in the field but not collected or remained in the sample were also recorded (especially Hemiptera and Coleoptera). The abundance using the same ranks as the Protocol 1 was recorded for all sample.

Each team applied both protocols in its sampling site. For the Protocol 2, once in the lab, the largest animals were picked up and identified first, and the rest were sorted using a stereoscope, with successive fractions of 50 individuals for riffles and pools samples separately. The total number of taxa from each fraction and habitat (riffles or pools) and the number of individuals per taxa were obtained.

Finally, the IBMWP, FBILL and IASPT were calculated for each team and protocol and the data was analyzed using hierarchical cluster methods and ANOVAs, after checking for normality. The statistical software used was Biodiversity-Pro (McAleece *et al.*, 1997) and Statistica (StatSoft, 1999). The different taxonomic experts in the project identified the individuals collected. The list of all taxa recorded is shown in Annex 1.

Riparian Vegetation

All the researchers applied the riparian vegetation index QBR, designed for Mediterranean streams with a previous training of its use made by the Barcelona research team which has designed the index (Munné *et al.*, 1998). This index has been successfully applied in several streams in Catalonia (Prat *et al.*1997, Prat *et al.* 1999) and in the Segura river basin (Suárez & Vidal-Abarca, 2000).

RESULTS

Macroinvertebrates: selection of a biological index

For each protocol and team the IBMWP (for riffles and pools) and FBILL (only riffles) were calculated. The data, presented in Figure 2, indicates that there are no differences in water quality for both indices using both protocols. It can be seen that all the teams have arose the "Very good biological quality" for the FBILL and IBMWP indices.

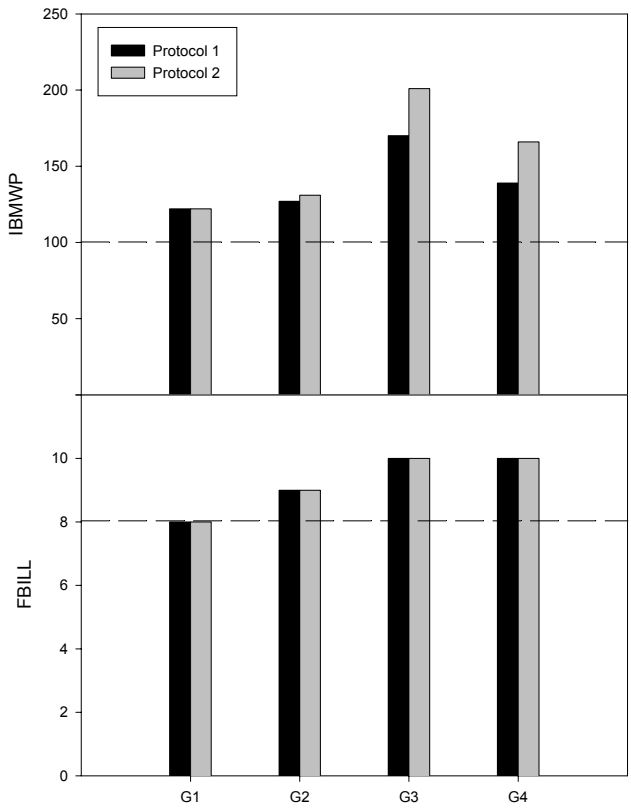

Figure 2. Values of IBMWP and FBILL for all sampling groups and both protocols. The IBMWP has been calculated using the community in the integrated sample (R+L) and the FBILL only in the lotic habitat (R). The discontinuous lines show the values of each biotic index and it can be considered a water quality of "Very good" (>100 in the IBMWP and between 8-10 in the FBILL) (G1, G2, G3 and G4=Groups 1, 2, 3 and 4).

Macroinvertebrates: effect of the sampling protocol

An analysis of the variance was performed to compare the number of families and the values of the IBMWP and IASPT, for both protocol and habitats, using each sampling team as replicates (n=4). According to the results, there are not significant differences between Protocol 1 and 2 in the IBMWP and IASPT indices (p=0.4884 and p=0.5924) (see Figure 3). Either, the total number of families found did not show differences between protocols (p=0.4832) or habitats (p(pools)=0.8351 and p(riffles)=0.7608).

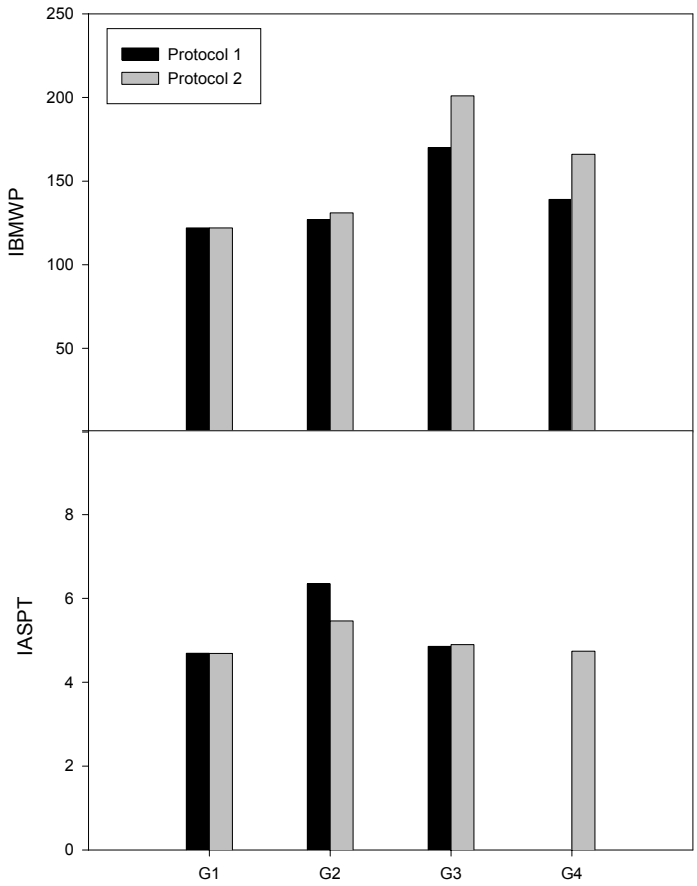

Figure 3. IBMWP and IASPT values following Protocols 1 and 2.

Although no significant differences were found between the number of families between both protocols, using the Protocol 2 a higher number of taxa was found in all the teams (Table 2), most of them small Diptera or Ostracoda, which where difficult to identify and to recognize in the field. The sampling team 2 was the one that observed a higher similarity between the numbers of taxa in both protocols.

Table 2. Families non-registered in field and found in the laboratory using Protocol 2, and their IBMWP values.

	Group	**SBMWP score**
Oligochaeta	G3	1
Ostracoda	G3	3
Lymnaeidae	G3	3
Caenidae	G1 and G4	4
Hydroptilidae	G3	6
Psychomyiidae	G3	8
Helodidae	G4	3
Sericostomatidae	G3	10
Elmidae	G3	5
Simuliidae	G4	5
Ephydridae	G4	2
Psychodidae	G4	4
Stratiomyidae	G2	4
Limoniidae	G3	4
Ceratopogonidae	G3	4

Macroinvertebrates: effect of the sampling team

Using presence/absence data of taxa found in the field and laboratory a cluster to check for similarities between sampling teams was performed. It was used the Jaccard index excluding the double absences (Figure 4). The major similarity between teams was found between groups 1 and 3 (50%), whereas team 2 was the most different (39% of similarity). However, the value of the IBMWP index in the field and lab of this team is close to those found by the other teams (Figure 3). The community found in team 2 although is poorer, has higher family scores, fact that could be related with a relative dominance of the riffle habitat in the reach sampled.

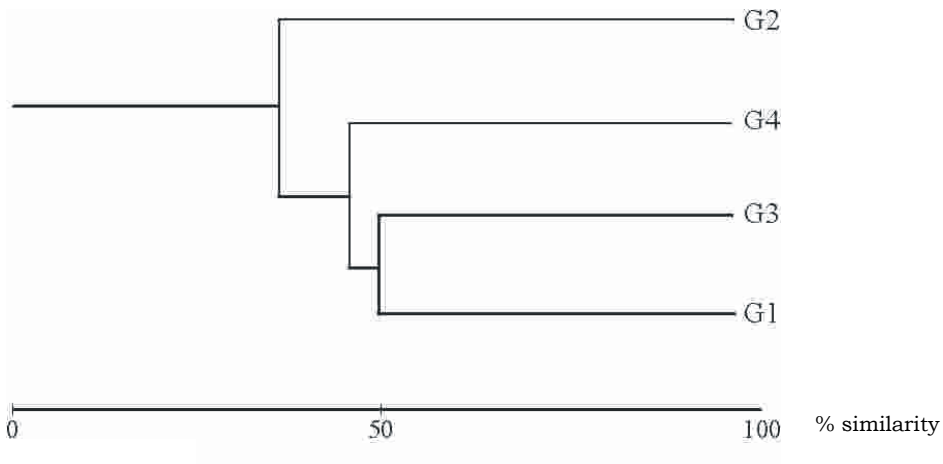

Figure 4. Dendrogram of the macroinvertebrate taxonomic composition found by eac Jaccard's method.

Macroinvertebrates: effect of the counted individuals

In Figure 5, number of taxa, values of IBMWP and IASPT indices calculated and accumulated for successive teams of 100 individuals in both habitats is plotted (50 from riffles and 50 from pools). There is an important increase in the number of families and the values of the IBMWP from 100 to 200 individuals counted, following a relative stabilization. For the IASPT, the value obtained for each team after a count of 100 individuals does not change much with an increase of the sorting effort. That would strengthen the use of this index, respect to the others, because of its conservative property. The analysis of the variance performed to test the differences between teams for all the variables indicated the presence of significant differences between teams ($p=0.000$ for the number of families; $p=0.000$ for the IBMWP and $p=0.008$ for the IASPT). According to that, the team 3 has a highest number of families and so a higher IBWMP value; however, the IASPT value is intermediate, indicating that the increase of families has been done with the addition of taxa in both habitats (riffles and pools), which should be related to the higher sampling effort in the stream reach for this team than the others. In the team 2, although there is a lower number of families and a lower IBWMP, the IASPT is higher than in the other teams, which is related with a dominant riffle habitat in the reach and higher individual scores of the macroinvertebrate families found.

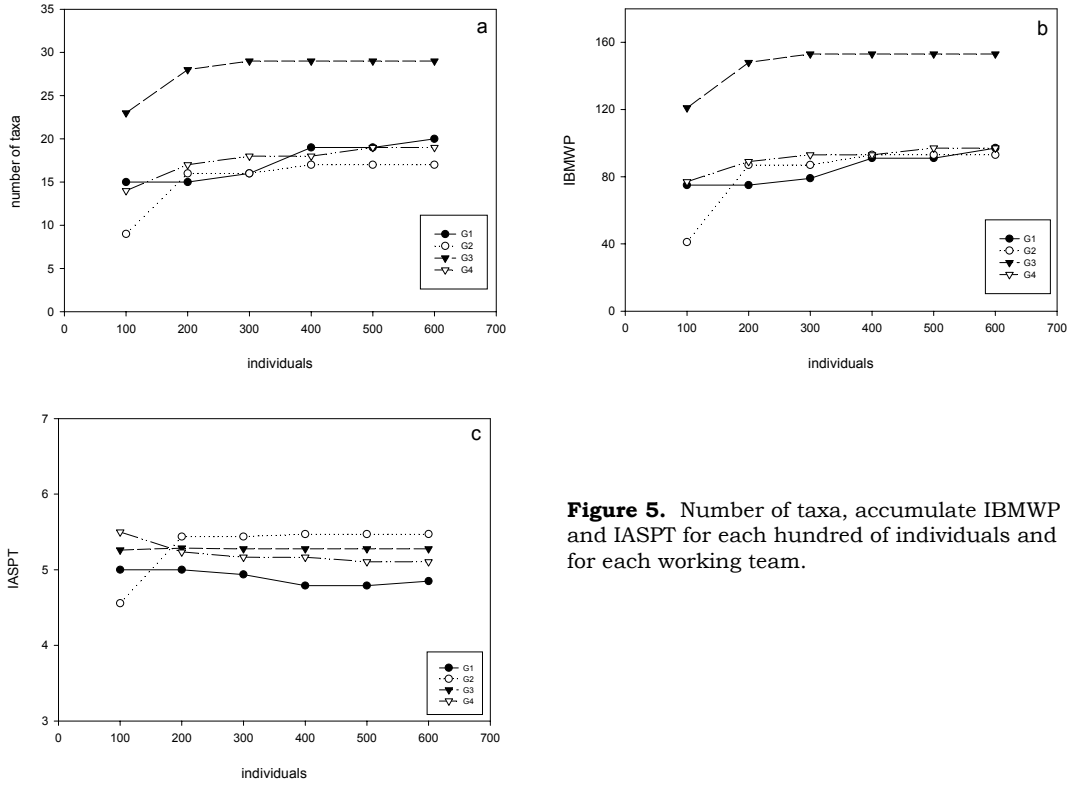

Figure 5. Number of taxa, accumulate IBMWP and IASPT for each hundred of individuals and for each working team.

The average values and the standard deviation of the number of taxa, IBMWP and IASPT indices for all teams and habitats are presented in Figure 6. It can be observed a sharp increase of the average values of each variable between the 100 and 200 individuals, although standard deviations are quite high. No significant differences were found in any case between the fractions of number of individuals counted (p=0.6912 for the number of families; p=0.7293 for the IBMWP and p=0.9788 for the IASPT). Consequently, a count of 100 individuals would be enough to get an optimum number of individuals, a IBMWP and IASPT indexes, respectively, although the high standard deviation present between groups.

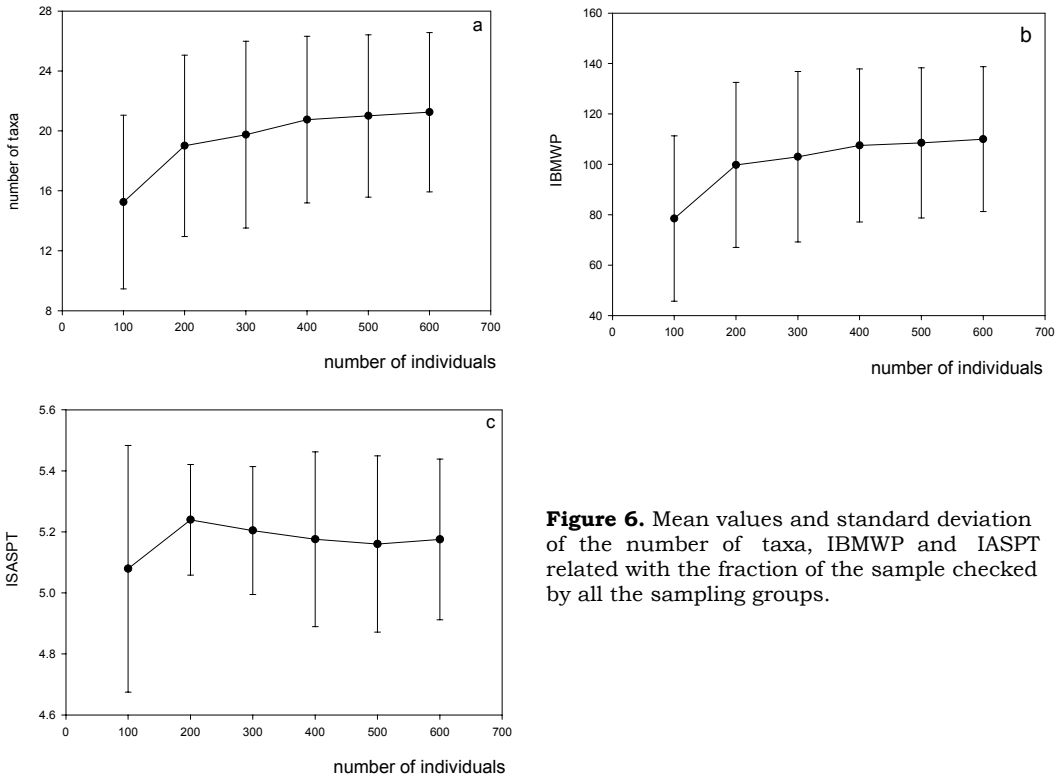

Figure 6. Mean values and standard deviation of the number of taxa, IBMWP and IASPT related with the fraction of the sample checked by all the sampling groups.

In Figure 7 differences between both habitats are shown. As in the other cases, it can be observed an asymptotic increasing of the number of taxa for each fraction of counted individuals and for both habitats. The analysis of variance performed to compare both habitats indicated significant differences between pools and riffles ($p<0.005$), with a higher diversity in the former. Moreover, with more individuals there is a tendency of a faster increase, in the taxa found in pools than in riffles. In Table 3, taxa exclusive for each habitat are presented. As can be expected, reophilic families as Simuliidae, Helodidae, Psychomyiidae, are only present in the lotic habitat, while Heteroptera, Odonata and Coleoptera are between the ones only found in the lentic samples.

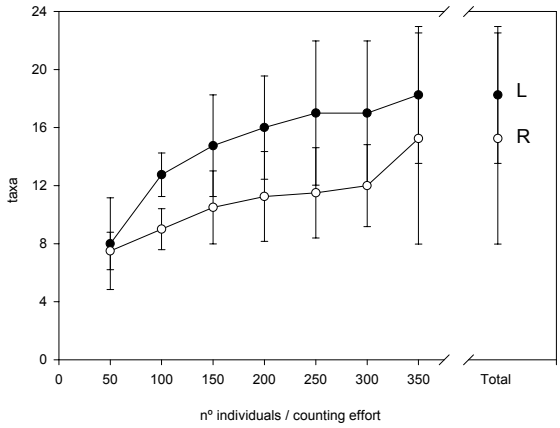

Figure 7. Number of taxa found related with the fraction of the sample checked by all the sampling groups, according to the lotic (R) or lentic (L) habitat.

Table 3. Exclusive taxa from lotic and lentic habitat.

Exclusive R	**Exclusive L**
Hydracarina	Coenagrionidae
Perlidae	Corduliidae
Aeshnidae	Cordulegasteridae
Helodidae	Dryopidae
Psychomyiidae	Haliplidae
Psychodidae	Hydraenidae
Simuliidae	Hydrophilidae
Stratiomyidae	Naucoridae
Tabanidae	Nepidae
	Notonectidae
	Polycentropodidae
	Ephydridae
	Glossiphoniidae

Riparian vegetation: effects of the sampling team

The analysis of variance performed to test differences in the QBR index between teams were significant ($p<0.001$). That could indicate two things: first, that the riparian vegetation is not uniform along the river, and therefore differences in QBR value are due to vegetation cover in the sampling reach. Another possibility is that index value disparities between reaches are due to insufficient training of the observers in the use of the index or linked to some subjectivity implicit in the index design allowing an observer effect (Munné *et al.*, in press). Although there

are some differences in the values between the observers in the same team, the changes in the quality ranks are at most of only one level (Table 4).

Table 4. QBR values by teams and observers.

Group	QBR value form each group member	Average
I	40 – 40 – 20 – 25 – 30	31
II	70 – 70 – 50 – 70 – 75	67
III	40 – 30 – 30 – 35 – 25	32
IV	40 – 45 – 55 – 60 – 55 – 50	50

DISCUSSION

The Rapid Bioassessment Protocols have been proved to be useful in wide regions (Resh & Jackson, 1993; Resh *et al.*, 1995). Several authors have studied the performance of RBPs when reducing the number of samples, the sampled area, the effort in the counting or the taxonomic resolution, and the implementation of the sorting in the field, (Resh & Unzicker, 1975; Stribling *et al.*, 1993; Marchant *et al.*, 1995; Resh *et al.*, 1995; Plafkin *et al.*, 1989; Barbour & Gerritsen, 1996; Barbour *et al.*, 1999; Smith *et al.*, 1999). The GUADALMED1 Project covers a large area that includes most of the watersheds draining into the Mediterranean Sea from the Iberian Peninsula (from the Besòs basin in Catalonia to the Guadalfeo basin in Granada), that is of about one thousand kilometers straight distance and an altitudinal ranges from the sea level to 4000 m in Sierra Nevada. In this case, a RBP must be adequate for the assessment of water quality in Mediterranean streams in Spain following the implementation of the WFD with care.

When a sampling methodology has to be implemented in a new area, firstly several protocols should be tested, and the most useful has to be selected (Rosenberg & Resh, 1993; Resh *et al.*, 1995; Hill *et al.*, 2000; Wrigth *et al.*, 2000). In the researched area, many previous studies have been done on macroinvertebrates and some detailed protocols already exist applicable to some catchments (Prat *et al.*, 2000). In the present study, two main sampling protocols currently used in Mediterranean rivers have been tested, one exclusively based in data got from the field and the other combining field and laboratory information. In both, a semi-quantitative sampling method has been applied using the kicking technique. In the literature

there is a large number of methods to collect macroinvertebrates according to the type of river and the objectives of the study (see Rosenberg, 1978; Elliot & Tullett, 1978, 1983). The kicking method is an easy one and has been recommended for biomonitoring surveys obtaining satisfactory results (Storey *et al.*, 1991; Metzeling & Miller, 2001). Moreover, an advantage of the non quantitative methods is that they can be used in substrates where the quantitative techniques are not applicable (Chessman & Robinson, 1987). The kind of data to be used (qualitative or quantitative) it is irrelevant to show community patterns, although at small scale or depending on the objectives to achieve, quantitative methods are need (Marchant, 1990).

Several authors suggest that the results obtained from combining samples from several habitats, give redundant information and therefore, one habitat could be enough to check for disturbance effects (Stribling *et al.*, 1993; Plafkin *et al.*, 1989). In mediterranean ecosystems, habitat availability may change naturally along the year, especially at the beginning of the drought period firstly with the lost of riffles and finally with the lost of pools (Gasith & Resh, 1999). As a consequence, protocols to be applied along year should be designed including both habitats. Thus, in these areas, as it has been suggested in other regions an integrated sample including lotic and lentic habitats could provide a better information about the river communities (Kerans *et al.*, 1992; Cuffney *et al.*, 1993), although it requires a greater sampling effort compared with the single-habitat methods.

Both indexes, IBMWP (Alba-Tercedor & Sánchez-Ortega, 1988) and FBILL (Prat *et al.*, 1999) have been shown to be properly applicable for assessing water quality in Mediterranean rivers. Moreover, several authors (Rico *et al.*, 1992; Prat *et al.*, 1997) showed high correlations between the FBILL or similar indices based in Trend index (Woodiwiss, 1964) and the IBMWP, indicating that both indices are useful for monitoring Iberian rivers. Because the importance of the lentic habitat in some mediterranean streams (mainly in summer) we discarded to apply FBILL index because it was designed to be applied only in runs and riffles and, therefore large part of the community may be lost using it. On the other hand, the IBMWP considers the sampling of both riffles and pools habitats and that is why it has been selected in the GUADALMED Project.

To assess the habitat diversity, Hannaford *et al.* (1997) compared the ability of students with and without training. The results were that the team with more experience had more precise results, far away from the other. In the same way, for macroinvertebrate identification, if the

results got in the field have to be compared with the laboratory ones, the experience and traineeship are important. Smith *et al.* (1999) found that qualified researchers identified in the field 76% of the families present in a sampling site, and 90% in the laboratory. Thus, this would imply that the use of sampling Protocol 1, although it is faster and effective, requires a previous effort of trainership, especially if the Protocol must be applied by the government monitoring program technicians (which may have low biology and ecology skills). In our study, where in each working team there were members of different centers with a similar experience identifying macroinvertebrates in the field, the differences between communities should be due to changes in the habitat sampled and not to the lack of experience. In fact, our results indicate that when differences were present, field values had a higher IBMWP and a lower IASPT what could be related to a major presence of the lentic habitat, or high IASPT with intermediate IBMWP, related to a dominance of riffles. However, despite these differences, there was a high similarity in the quality rank of the IBMWP produced for each team.

Usually the number of taxa found in a sample increase asymptotically, when the effort of sorting and counting increases (Courtemanch, 1996; Vinson & Hawkins, 1996). That fact has produced strong arguments against the fixed counting method to assess the sample diversity (Cuffney *et al.*, 1993; Countermanch, 1996; Vinson & Hawking, 1996), although other studies show its usefulness in biomonitoring (Plafkin *et al.*, 1989; Barbour & Gerritsen, 1996; Tiller & Metzeling, 1998; Barbour *et al.*, 1999; Metzeling & Miller, 2001). According to our results, a fraction of 100 individuals would be enough to get an optimal number of families, and IBMWP and IASPT rank quality, after removing the largest organisms. However (Figure 5), both the number of taxa and IBMWP increases between 100 and 200 individuals in all the teams, although it is not significant because the high standard deviation. We consider that to get a safer IBMWP value and a more complete list of taxa, 200 individuals are required for routine monitoring in Mediterranean streams. Even though the value of IBMWP would increase using more than 200 individuals, the quality rank would remain the same (Alba-Tercedor & Sánchez-Ortega, 1988), indicating that the 200 individuals are enough to get a significant and representative quality rank even at lower standard deviation values. On the other hand, the IASPT seems to be stable even from 100 individuals (Figure 5), as it is a more conservative metric.

When both sampling protocols were compared, there were not significant differences between them in the number of families, and the IBMWP and IASPT indexes. Thus, the selection of one or the other does not affect the results in terms of water quality. However, in reference

conditions the community is always more diverse (Reynoldson *et al.*, 1997) and the use of the Protocol 1 would imply a loss of information because the rare species or the smallest ones would not be detected. Therefore, because the data of references sites may be used not only for the biological quality classification (but also for other purposes, as to design of a RIVPACS-type assessment method) for the Guadalmed project it was agreed to use the Protocol 2.

In Marchant *et al.* (1995) and Marchant (1999) it has been suggested that the exclusion of the rare taxa does not imply a loss of ecological information. However, other studies (Cao *et al.*, 2001) demonstrate the importance of the rare species to get a good assessment of the water quality (Cao & Williams, 1999). In our study, although there are not differences between the IBMWP and IASPT determined by all the sampling teams (but note the high standard deviation), when the data for each team is analyzed separately the number of individuals required to stabilize the IBMWP and the IASPT are 200 and 100, respectively. This difference is due to the presence of rare taxa that let to a IBMWP increase, although the IASPT would remain stable. The rare taxa (with low abundance and frequency, and usually very small) found between 100 (optimum for IASPT) and 200 (optimum for IBMWP) individuals are important because they imply a significant increase of the IBMWP. The rare species would be the key species to assess the effects of the disturbances at a specific level, because usually they present narrow ecological niches (Cao & Williams, 1999). For instance, the Beraeidae family (low abundance and frequency) has a score of 10 in the IBMWP and its presence is limited to the small streams with mosses, gravel or sand. Consequently, after counting 200 individuals, as the number of taxa increase slightly without increasing IBMWP or IASPT rank, we suggest checking the rest of sample only for new and rare taxa that might provide extra information useful for specific studies.

Barbour & Gerritsen (1996) showed that counting between 100 and 300 individuals is enough to discriminate significantly different ecological patterns. However, the exact number required can vary between areas (Barbour & Gerritsen, 1996) and sites (Vinson & Hawkins, 1996). For instance, Carter & Resh (2001) presented how in the different states of North America the number of individuals counted differs from 100 to 500. According to our invertebrate exercise, made in a reference site, 200 individuals (after removing large animals) is a good number to assess biological quality in Mediterranean streams which is in the range applied in other countries (Carter & Resh, 2001).

The more appropriate taxonomical level to the assessment of the water quality has been highly discussed (Resh & Unzicker, 1975; Cranston, 1990; Marchant *et al.*, 1995; Bowman & Bailey, 1997). Using a lower taxonomical resolution implies a better precision and information (Furse *et al.*, 1984; Resh *et al.*, 1995), although the number of studies and biotic indices that use the family to assess water quality are large because of its simplicity and cost-efectiveness (Armitage *et al.*, 1987; Alba-Tercedor & Sánchez-Ortega, 1988; Corkum, 1989; Prat *et al.*, 1999, Hewlett, 2000). However, Stubauer & Moog (2000) point out the use of biological indices at family level could imply a loss of information about the environmental effect of the disturbance. Several studies shown that using higher taxonomical levels as families, the distribution patterns of the communities are similar than using the species level (Furse *et al.*, 1984; Ferrano & Cole, 1992; Rutt *et al.*, 1993; Marchant *et al.*, 1995; Zamora-Muñoz & Alba-Tercedor, 1996; Bowman & Bailey, 1997; Nielsen *et al.*, 1998). The IBMWP index, used in the GUADALMED methodology, uses the family level, and numerous studies in the Iberian Peninsula indicate its utility to detect disturbances (Zamora-Muñoz *et al.*, 1995; Alba-Tercedor, 1996; Zamora-Muñoz & Alba-Tercedor, 1996; García-Criado *et al.*, 1999; Alba-Tercedor & Pujante, 2000). On the other hand, Bowman & Bailey (1997) comparing similarity matrixes using the genus and the family level, found that the correlation between both levels in the disturbed sites are higher than in the reference ones. Bonada *et al.*, (2001) using the caddisfly community of the GUADALMED Project identified at the species or genus level, found that the general patterns are similar with those shown by the family level.

The QBR results indicate that there are differences between the values found in close sampling sites, which shows the dependency of the index from the local conditions as was pointed out in Munné *et al.* (1998). This peculiarity of the index is very important in the reference sites, where according to the WFD, these sites should have a very good ecological status, including riparian vegetation. Thus, the QBR method is a useful RBP to evaluate the status of the riparian vegetation. Although the index may be subject to some over or under evaluation when it is applied by several observers, values of quality do not change very much and the results improve with training (Munne *et al.*, in press). The method has been used by all the GUADALMED teams and results are published elsewhere (Suarez *et al.*, in press).

In summary, according to our exercise, to obtain the best results in the assessment of ecological status in the Mediterranean area, the protocol to be used will be different if the site is a reference station or not. In a reference site and to evaluate its biological quality, the Protocol 2 should be used counting and identifying until 200 individuals in the lab to avoid

differences between basins and habitats, after sorting the biggest individuals (Cuffney *et al.*, 1993; Vinson & Hawkins, 1996). The use of Protocol 2 allow us to keep the samples to be analyzed until lowest taxonomic resolution, and made possible studies to look for patterns in the distribution of some taxa in the Mediterranean area (Bonada *et al.*, 2001). On the other hand, in the no-reference sites, the use of the Protocol 1 will be enough to provide data for biological index determination because the community is poorer, abundant and easier to identify (Countermanch, 1996). In both cases, combined samples from both habitats (riffles and pools) will be required. The use of Protocol 1 may simplify the routine analysis performed by water authorities and allows to improve the effectiveness per sampling site and even to increase the number of sites to be monitored. However, we understand that the use of Protocol 1 it should be only applied when the objective of the study is to assess biological quality, whereas in other cases (e.g., when studies about biogepographical distribution patterns of several taxa) Protocol 2 is needed to obtain the maximum information without biasing the results.

The intercalibration and selection of a sampling protocol to assess streams and rivers ecological status is an important step to take into account before starting any biomonitoring program in a wide area, because not all the methods are equally applicable (Rosenberg & Resh, 1993; Resh *et al.*, 1995; Hill *et al.*, 2000; Wrigth *et al.*, 2000). Moreover, the objectives of those methods could be different from ours (Barbour *et al.*, 1999). The WFD requires assessing the ecological status using biological criteria, and so protocols based in this idea have to be implemented in Europe. These protocols must be easy to apply and cost-effective, as they will be used by the administration although a minimum of training is required (Hannaford *et al.*, 1997) to get optimal results.

GUADALMED RBP PROTOCOL

Protocol 1. Non-reference sites
Only for biological assessment studies

- Sampling all the available habitats in a 100 m reach.
- Kicking method with a mesh size of 250 μm, removing all the substrate upstream of the net.
- For the riffle habitats, locate the net in front of the rock, remove the substrate and clean well the rocks.
- For the pool habitats, sweep the bank vegetation, and remove the gravel substrate of the pools.
- Before the net is clogging, put the collected material in plastic white trays.
- Check often the different taxa found in the tray and identify. Record them in the field sheet with a abundance rank: 1 (1-3 indv.), 2 (4-10 indv.), 3 (10-100 indv.) or 4 (>100 indv.).
- Keep in vials with alcohol 70% the taxa difficult to identify on the field.
- Repeat the sampling process until no more new taxa is observed.
- Identify in the lab the taxa collected.
- Calculate the biotic index IBMWP and IASPT using all taxa found in the field or in the lab for both habitats.

Protocol 2. Reference sites
Depending on the objectives, non-reference sites should be also sampled using Protocol 2

- Sampling all the available habitats in a 100 m reach.
- Kicking method with a mesh size of 250 μm, removing all the substrate upstream of the net.
- For the riffle habitats, locate the net in front of the rock, remove the substrate and clean well the rocks.
- For the pool habitats, sweep the bank vegetation, and remove the gravel substrate of the pools.
- Put all the contents of the net in a plastic white tray and take a quick look to identify major taxa. Put the material in a labeled plastic jar with alcohol 70%, or formol 4% for the lentic and lotic habitats, separately.
- Repeat the sampling process to check only for non-collected taxa.
- Bring the samples to the lab. Sort the biggest invertebrates with forceps, identify them.
- Using a stereoscope, sort 200 individuals of the sample, identify and record the abundance of all sample: 1 (1-3 indv.), 2 (4-10 indv.), 3 (10-100 indv.) or 4 (>100 indv.).
- Check the rest of sample looking for new taxa not found and record their abundance.
- Calculate the biotic index IBMWP and IASPT using all taxa found in the field and the lab for both habitats.

For all sites
- Sampling 4 times per year: spring, summer, autumn and winter.
- Measure temperature, pH, discharge, conductivity and oxygen with field devices.
- Collect a water sample to analyze the chemical parameters established in the WFD.
- Measure the QBR index using the field sheet.

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Annex 1. List of taxa found in the sampling site.

OLIGOCHAETA	
ACHAETA	Erpobdellidae
	Glossiphoniidae
TURBELLARIA	Planariidae
CRUSTACEA	*Echinogammarus* sp.
MOLLUSCA	*Lymnaea truncatula*
	Lymnaea peregra
	Physella acuta
	Hydrobiidae
	Potamopyrgus jenkinsi
EPHEMEROPTERA	*Alainites muticus*
	Baetis pavidus
	Baetis rhodani
	Cloeon dipterum
	Procloeon bifidum
	Caenis luctuosa
	Ecdyonurus gr. *ruffi-wautieri*
PLECOPTERA	Nemouridae
	Perlidae
COLEOPTERA	*Nebrioporus clarki*
	Deronectes hispanicus
	Bidessus minutissimus
	Graptodytes fractus
	Agabus gr. *brunneus*
	Agabus ddymus
	Agabus biguttatus
	Hidroporus discretus
	Lacophilus hyalinus
	Lacophilus minutus
	Haliplus lineatocollis
	Haliplus mucronatus
	Anacaena limbata
	Anacaena globulus
	Helochares lividus
	Laccobius gracilis
	Laccobius hispanicus
	Ochthebius quadrifoveolatus
	Ochthebius marinus
	Limnebius maurus
	Limnius volkmari
	Dryops gracilis
	Pomatinus substriatus
	Helophorus flavipes
	Elmis mauguetti
	Hydrocyphon sp.
	Elodes sp.
ODONATA	*Pyrrhosoma nymphula*
	Orthetrum coerulescens
	Orthetrum cf.
	Cordulegaster annulatus
	Onychogomphus forcipatus
	Onychogomphus uncatus
	Anax imperator
	Boyeria irene
HETEROPTERA	*Naucoris maculatus*
	Notonecta maculata
	Sigara nigronileata
	Nepa cinerea
	Microvelia pygmaea
	Hydrometra stagnorum
TRICHOPTERA	*Rhyacophila* gr. *munda*
	Agapetus sp.
	Hydropsyche gr.
	Stenophylax sp.
	Plectrocnemia sp.
	Tinodes waeneri
	Sericostomatidae
	Mesophylax aspersus
	Hydroptila vectis
DIPTERA	Tanypodinae
	Tanytarsini
	Orthocladiinae
	Corynoneurinae
	Quironomini
	Athericidae
	Limoniidae
	Dixidae
	Psychodidae
	Tipulidae
	Ceratopogonidae
	Tabanidae
	Simuliidae
	Stratiomyidae
	Ephydridae