Impact of a fluoropolymer plant on a river ecosystem: evaluation using a combined chemical, ecological and genetic approach

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Abstract

Effect-based monitoring is a recommended approach suggested in European Guidelines to assess the response of ecosystem affected by a pollution source, considering the effects at community, population, individual but also at suborganism level.

The present paper reports the application of this approach to the assessment of the impact of the discharge of a fluoropolymer plant on the macrobenthic community of a Northern Italian river: river Bormida (Piedmont region). The macrobenthic community living downstream the industrial discharge has been exposed for decades to high perfluorooctanoic acid (PFOA) concentrations coming directly from the discharge, unlike the community living upstream.

Impact assessment was performed by comparing the populations living up- and downstream of the discharge (respectively sampling site U and D). Macrobenthic samples were collected in both sites in three seasons in 2012 (June, August and October). Ecological quality, evaluated by Italian official national classification system, and the community structures were evaluated for all the samples. The AFLP (Amplified Fragment Length Polymorphism) genetic technique was applied to genotype forty individuals of Hydropsyche modesta (Trichoptera) collected in the two sampling sites in June and August.

The two communities show no differences in ecological quality and slight differences in the community structure. At the genetic level, percentage of variation between the two populations was 6.8% (F_{ST} =0.06791) consistent with a genetic pressure applied on the downstream population. Nevertheless no significant differences, compatible with the presence of selective effects, were highlighted.

Keywords: PFOA; effect-based monitoring; AFLP; river pollution; genetic selection.

1. Introduction

Protection of water bodies from chemical pollution is generally based on analysis of a priori selected list of compounds and comparison of measured concentrations with compound-specific quality objectives. Chemical monitoring is therefore usually focused on known and regulated substances that are known to pose a threat to or via the aquatic environment. There are two main limitations of this approach: the list of substances normally never covers all the substances actually present in the monitored water body and the possible effects caused by the mixture of the chemical substances are not taken into account. A complementary approach is to study

the overall response of ecosystems to multiple, bioavailable and often unknown chemicals at different levels of biological organisation, such as community, population, individual and suborganism. This approach has been recently reviewed and recommended also in an European Guideline on effect-based monitoring (European Commission, 2014) also in term of assessment of water body quality in the framework of European regulation. It is generally accepted that the combined approach of chemical and biological monitoring is highly effective for assessing the impact of specific sources of pollution on a water body. The present paper reports the application of the combined approach of chemical, ecological and genetic analysis to the assessment of the impact of the discharge of a fluoropolymer plant on the macrobenthic community of a Northern Italian river. This plant, located in Spinetta Marengo, next to Alessandria (Piedmont, Northern Italy), and discharging into river Bormida, a tributary of river Tanaro, has been recognized as the most significant point source of PFOA in the river Po basin, being the PFOA load, carried out by the river Po to the Adriatic Sea (about 2.1 t y-1), mostly due to this single source (Loos et al., 2008; Valsecchi et al., 2014).

It is known that fluoropolymer plants are very significant sources of poly- and per-fluorinated compounds for the aquatic environment (Prevedouros et al., 2006). Production plants of polytetrafluoroethylene (PTFE) are the most important source of perfluorooctanoic acid (PFOA) which is used as an emulsifier in aqueous solution during the emulsion polymerisation of tetrafluoroethylene. PFOA is not consumed during the polymerization process and remaining compound may be released into air and water, (OECD, 2006). In 2006 PFOA point source emissions from fluoropolymer manufacturing have been reported to be approximately 60% of the total PFOA emitted with 23%, 65%, and 12% distributed to air, water, and land, respectively (Prevedouros et al., 2006).

Few studies have been carried out to describe the impact of fluoropolymer plants on the surrounding environment, but the aim was generally to study the distribution of the emitted perfluorinated compounds (PFAS) into the different environmental compartments, especially air, groundwaters and raw water resources (Davis et al., 2007; Dauchy et al., 2012a; Dauchy et al., 2012b) or workers' and residents' blood (Bao et al., 2011). No extensive study has been performed on the impact of the plant emission on the aquatic ecosystem.

Emitted primarily into water, PFAS accumulate in various environmental compartments, because of their special characteristics such as resistance to hydrolysis, photolysis, microbial degradation (Giesy and Kannan, 2001; Prevedouros et al., 2006). Aquatic organisms are exposed by water or food to a range of PFAS and this possibly results in adverse effects. Despite this concern, data about the physicochemical properties and aquatic toxicity of PFASs are still limited, though some reviews which collected data on toxicity of PFAS on aquatic organisms have been already published (Giesy et al., 2010; Hoke et al., 2012; Ding and Peijneburg, 2013). Although results showed that currently known PFAS levels in surface water have no acute harmful impact on aquatic organisms, being predicted no-effect concentrations (PNECs) in the range from 10 to 100 µg L-1 (Hoke et al., 2012), some PFASs (including the mostly studied perfluorooctanesulphonic acid, PFOS) are suspected to have long-term adverse effects on aquatic organisms (Ahrens, 2014). Current testing requirements are not sufficient to identify effects such as immunotoxicity and endocrine disruption, which have been linked to PFAS exposure (Scheringer et al., 2014; Grandjean and Budtz-Jørgensen, 2013; White et al., 2011). Polyfluoroalkyl and perfluoroalkyl substances are continuously introduced into aquatic ecosystems and are ubiquitously present in complex mixtures. However, little is known about the interactive toxicity of PFAS mixtures at environmentally relevant concentrations or about interactions with other natural and anthropogenic stressors. In addition, because exposure to PFASs is continuous, further information about their ecotoxicological potential in multiple

generations, species interactions should be useful to assess the risks for PFASs to affect aquatic ecosystem structure and function (Ahrens 2014, Stefani et al., 2014).

The aim of the present work is to study the effects of an important fluoropolymer plant on the aquatic benthic community living in the river downstream the discharge of an important fluorochemical industry. We compared the ecological quality of the communities living up- and downstream the discharge and, in order to highlight the possible sub-lethal effects of the industrial discharge, we tested the presence of effects at genetic scale on native populations.

2 Study Area

The river Bormida is 154 km long with a catchment area of 2609 km2 which extends between the Liguria and Piedmont and is the main tributary on the right bank of the river Tanaro. Few kilometers after receiving the river Bormida waters, the river Tanaro flows into the river Po, the main river in the Northern Italy.

The studied stretch of the Bormida is located at Spinetta Marengo, next to Alessandria (Piedmont, Northern Italy) where a fluoropolymer plant is present. The plant, which produces fluoropolymers and fluoroelastomers, is very close to the right bank of river Bormida and discharges the process waters after treatment directly into the river (coordinates WGS 84: +44° 53' 33.05"N, +8° 38' 35.81"E). The chemical plant was founded in 1905 for the production of superphosphate fertilizers and then produced also chromates and dichromates. In the eighties the chemical plants were converted to the production of fluorinated polymers. Nowadays the plant produces a wide range of fluorinated specialties including polymers, elastomers, fluids and coatings. Since many years the most important product is polytetrafluoroethylene (PTFE). The Solvay Company signed the 2010/2015 PFOA Stewardship Program to formally put a stop to environmental release of PFOA and its related compounds within 2015 (US-EPA, 2006) and recently optimised the polymerisation processes also testing non-fully fluorinated alternative surfactants, such as the proprietary functionalized polyfluoropolyethers (Wang et al., 2013). Other important products of the plant are perfluoropolyether polymers, sold under the Solvera® and Fluorolink® brand names, whose production capacity has been progressively increased during 2008.

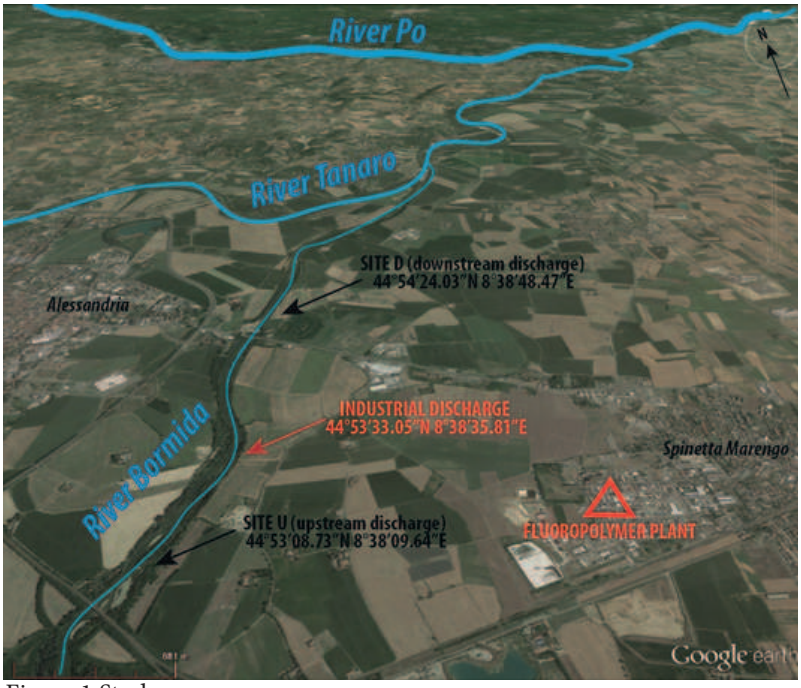

Figure 1 Study area

In order to study the impact of the discharge of the fluoropolymer plant, two sampling points were sited along the river up- and downstream (Figure 1, Table 1) the discharge of the industrial area of Spinetta Marengo,

These two stations have been also used by the Regional Environmental Agency (ARPA Piemonte) for the operational monitoring for classification according to the Water Framework Directive (2000/60/EC, WFD). In the 2009-2011 period the ecological classification was 'moderate' for both sites (http://www.arpa.piemonte.it/reporting/indicatori-on_line/componenti-ambientali/acqua_fiumi-stato-ecologico), while the chemical

Table 1 Sampling points

	Site name	Latitude	Longitude
Upstream industrial discharge	U	44°53'08.73" N	8°38'09.64" E
Downstream industrial discharge	D	44°54'24.03" N	8°38'48.47" E

quality class was 'good' (http://www.arpa.piemonte.it/reporting/indicatori-on_line/componenti-ambientali/acqua_fiumi-stato-chimico), based on the compliance with Environmental Quality Standards for priority substances.

3 Materials and methods

3.1 Chemical Monitoring

Water monitoring was carried out from 2008 to 2014 with a monthly frequency during the period of the sampling of the biological community (2012). On May 2012 sediments were also sampled in the two monitoring stations.

Water samples were collected in polypropylene (PP) vials directly from the bank or using a bucket in case of not accessible bank. Bed sediments were collected from the upper 5 cm using a grab sampler and stored in pre-cleaned glass bottles. Water temperature and dissolved oxygen were measured in field during the sampling by portable probes. All samples were collected in PP centrifuge tubes and refrigerated at 4 °C until analyses, which were carried out within five days. In laboratory pH, conductivity and main ionic composition were measured. NO3-, SO42- and Cl- were analysed by ion chromatography using a Dionex LC25 equipped with an AS11 column and 30 mM KOH eluent. Ca2+, Mg2+, Na+ and K+ were analysed by a Dionex ICS2000 ion chromatograph with a CS12 column and 20 mM methansulphonic eluent.

In this study, we targeted 12 pefluoroalkylacids (PFAA): perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), perfluorododecanoic acid (PFDoDA), perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS) and perfluorooctane sulfonate (PFOS). For the analysis of PFAA, water samples were centrifuged and analysed by on-line-SPE-UHPLC-MS/MS according to the method reported in Castiglioni et al. (2015). A Thermo EQuan system with a polar endcapped C18 pre-concentration column (Thermo Hypersil Gold aQ 12 µm, 20 × 2.1 mm), and a Thermo Hypersil Gold PFP analytical column (1.9 µm, 50 × 2.1 mm) were used for on-line extraction. Before injection, standards and samples were acidified to pH 3 and spiked with stable isotopically labeled (SIL) internal standards. Five mL of centrifuged and acidified sample were loaded into the pre-concentration column; the trapped analytes were eluted from the pre-concentration column to the analytical column by a gradient of 2 mM CH3COONH4 (5% methanol) and methanol at 300 µL min-1. A QqQ mass spectrometer (Thermo TSQ Quantum Access MAX) equipped with a heated-electrospray ionisation (Thermo HESI-II) probe was used. The mass spectrometer operated in the negative SRM mode. Quantification was done using the isotopic dilution method and calibration curves were made before each analytical run. Validation data can be found in Castiglioni et al., 2015. Briefly, the on-line SPE-UHPLC-MS/MS method gave recoveries between 87 and 134 % with limits of detection (LODs) ranging from 0.2 to 5.0 ng L-1 and LOQs from 1 to 20 ng L-1. The performance of the on-line-SPE-UHPLC-MS/MS method was verified

in the "PFOS and PFOA in surface water" proficiency test organised in 2013 by the PT-WFD network (http://www.pt-wfd.eu). Analytical results were satisfactory with calculated z-scores of +1.43 and +1.70 respectively for PFOS and PFOA.

Fresh sediments were extracted by sonication with acetonitrile spiked with the SIL solution. The extracts, after centrifugation and reduction to 1 mL volume under gentle nitrogen stream and filtration, were injected in the UHPLC-MS/MS and analysed by using the same analytical method of the water samples.

3.2 Ecological monitoring

Benthic macroinvertebrates were collected using artificial substrates (ASs). This kind of sampling method is usually performed where the access to the river is difficult or dangerous and sampling with Surber is not always possible, and is carried out in Italy according to the current standard procedures for the ecological classification under Water Framework Directive (Buffagni et al, 2007). Each AS consisted of ten 10x10 cm wooden boards mounted on a threaded rod and had a total surface of 0.1 m2. Five of these ASs formed a single sampling unit with a total sampling surface of 0.5 m2. Each sampling unit was deployed in the water column about 1 m below water surface for one month at sites U and D. Two sampling units were exposed in each sampling site in three different seasons in 2012 (May, August, October). After one month, the ASs were retrieved from the water and all invertebrates colonizing the structure were recovered and carried to the laboratory for taxonomic identification and evaluation of the community structure.

Identification of invertebrates was carried out to genus/species level at stereomicroscope using taxonomic keys). Ecological quality was calculated on the basis of the combination of STAR_ICMi (Buffagni et al., 2007) and MTS (Buffagni, 1997) values according to the Italian official classification system in use when sampling is by means of artificial substrates (DM 260/2010). For the calculation the MacrOper.ICM 1.0.5 software (Buffagni and Belfiore, 2011) was used.

Principal Component Analysis (PCA) was performed (Statistica 8) to verify the differences between Sites U and D using the data relating the community and physico-chemical data described in Figure 2. "Rare" taxa, whose abundance is less than three individuals, were excluded from the analysis.

3.3 Genetic analysis

Ten individuals of *Hydropsyche modesta* (Trichoptera) were selected in each sampling site for two sampling campaigns (May 2012, August 2012). This species has been selected because it was found in both sites at sufficient abundance to carry out the genetic analysis. DNA from abdominal tissue of a total of 40 individuals was extracted using ArchivePure DNA Cell/Tissue Kit (5 PRIME). The integrity and concentration of extracted DNA was then tested on 1% agarose gel electrophoresis.

The AFLP technique (Vos et al 1995; Vos and Kuiper 1996) has been applied to estimate genetic diversity. As suggested and tested by Ajmone-Marsan et al (1997), the endonuclease *Eco*RI and *Taq*I were used respectively as rare and frequent cutter of the genomic DNA. To produce AFLP markers the operating protocol has been borrowed from Clarke and Meudt (2005) and Ajmone-Marsan et al (1997): ~250 ng of DNA was incubated 90 minutes at 65 °C with 2.5 units *Taq*I (Promega), 1 µl of Multi-Core buffer 10X, 0.1 µl bovine serum albumin (BSA) in a total volume of 10 µl. In the next step 6 µl of a solution having the same amount of 10X Multi Core Buffer, BSA and 3 unit of *Eco*RI (Promega) was added and the resulting 16 µl were incubated 90 min at 37°C and 15 min at 70 °C. To ligate adapters, 20 µl of a solution containing 5 µl digested DNA, 100 pmol *Eco*RI

adapters, 100 pmol *Taq*I adapters, 2 µl 10X Ligation Buffer and 3 unit T4DNA ligase (Promega) were incubated for 3 h at 37 °C. Ligations reactions were stored at -80 °C.

1 µl of ligated DNA was then added to a PCR reaction mix containing PCR Buffer 1X, 0.5 µM of each primer carrying one selective nucleotide (Table 2), 350 µM of total dNTPs, 0.5 unit of AmpliTaq polymerase (5 PRIME) in a total volume of 20 µl. Samples were subjected to 20 cycles of (pre)amplification with the following profile: 30 sec at 94 °C, 1 min at 56 °C and 1 min at 72 °C, followed by 10 min at 72 °C for the completion of partial amplifications. The preamplified template was diluted 30-fold with MilliQ water (Merck Millipore) and processed further.

1 µl of diluted preamplified template was added to a PCR reaction mix containing PCR Buffer 1X, 1.5 mM $MgCl_2$, 350 µM of total dNTPs, 0.5 µM each of primers terminally fluorescent FAM- or HEX-labeled (*Eco*RI primers) or unlabeled (*Taq*I primer) carrying three arbitrarily chosen selective nucleotides each, 0.5 unit of PerfectTaq DNA Polymerase (5 PRIME) in a total volume of 20 µl. Amplification was performed with the following profile: 2 min at 94 °C; 10 cycles of 30 sec at 94 °C, 30 sec at 65 °C (annealing temperature was reduced by 1 °C/cycle), 1 min at 72 °C; 30 cycles of 30 sec at 94 °C, 30 sec 56 °C, 1 min at 72 °C followed by 30 min at 72 °C. The touchdown PCR promotes high-stringency amplification. All the sequence of adapters and primers used in the experiment are in Table 2.

Genotyping was performed by using an ABI 3730XL (Applied Biosystems) sequencer and fragments reading was done manually by using the Peak Scanner v. 1.0 freesoftware (Applied Biosystems).

All unambiguously identified peaks, including monomorphic peaks between 50-500 bp, and the scoring results were exported as a presence/absence matrix.

Parameters of genetic variability were calculated with the ARLEQUIN 3.5 software (Excofier and Lischer, 2010). Analysis of molecular variance (AMOVA) was carried out to test the presence of genetic structure induced by contaminant pressure or by temporal shift of genetic variability occurred during the sampling season. Two different group settings were tested: 1) June vs. August sampling; 2) upstream vs. downstream population respect to the contaminant discharge.

The genetic divergence (F_{ST}) between pairs of populations was also calculated. Moreover we reconstructed the Minimum Spanning tree (MS-tree) between all the individual haplotypes of H.modesta, based on a matrix of squared Euclidean distances, by using FigTree 1.4 (http://tree.bio.ed.ac.uk/software/figtree).

Test for the presence of selection was performed with the software BayeScan version 2.1 (Foll and Gaggiotti,

Table 2 Sequence of adapters and primers used in the experiment

	Name	Sequence	Label
Adapters *Eco*RI	*Eco* top strand	5'-CTCGTAGACTGCGTACC	
	Eco bottom strand	5'-AATTGGTACGCAGTCTAC	
Adapters *Taq*I	*Taq* top strand	5'-GACGATGAGTCCTGAC	
	Taq bottom strand	5'-CGGTCAGGACTCAT	
Primers *Eco*RI	E01	5'-GAC TGC GTA CCA ATT CA	
	E32	5'-GAC TGC GTA CCA ATT CAA C	Fam
	E00	5'-GAC TGC GTA CCA ATT CAC C	Hex
Primers *Taq*I	T01	5'-GAT GAG TCC TGA CCG AA	
	T02	5'-GAT GAG TCC TGA CCG AC	
	T32	5'-GAT GAG TCC TGA CCG AAA C	
	T49	5'-GAT GAG TCC TGA CCG ACA G	

2008), which exploits a Bayesian inference method to directly estimate the posterior probability (PP) for each locus to determine a higher or lower than average level of genetic divergence between populations. These loci behave as outliers, and may be considered as under directional or balancing selection respectively. A logistic regression model is used in the model, in which each $F_{ST\ (i,j)}$ for locus i in population j is decomposed as a linear combination of a locus (α_i) and a population effect (β_i), respectively. A significant positive value of αi suggests the presence of adaptive selection, whereas a negative value suggests stabilizing selection. Standard settings, as suggested by Foll and Gaggiotti (2008), were used in the analysis.

4. Results

4.1 Site characterisation

In order to understand if the differences in the physico-chemical characteristics between the two sites can influence the biological communities, we compare physico-chemical data collected between 2008 and 2014 in the two sites. There are no significant differences (t-test; $p<0.05$) between the two sites for temperature, dissolved oxygen and pH (Figure 2), while the average of conductivity data in the Site D is about 200 µS cm^{-1} higher than in Site U. By the analysis of the main ionic composition, the higher value in the downstream site is mainly due to the higher concentrations of chloride and sodium.

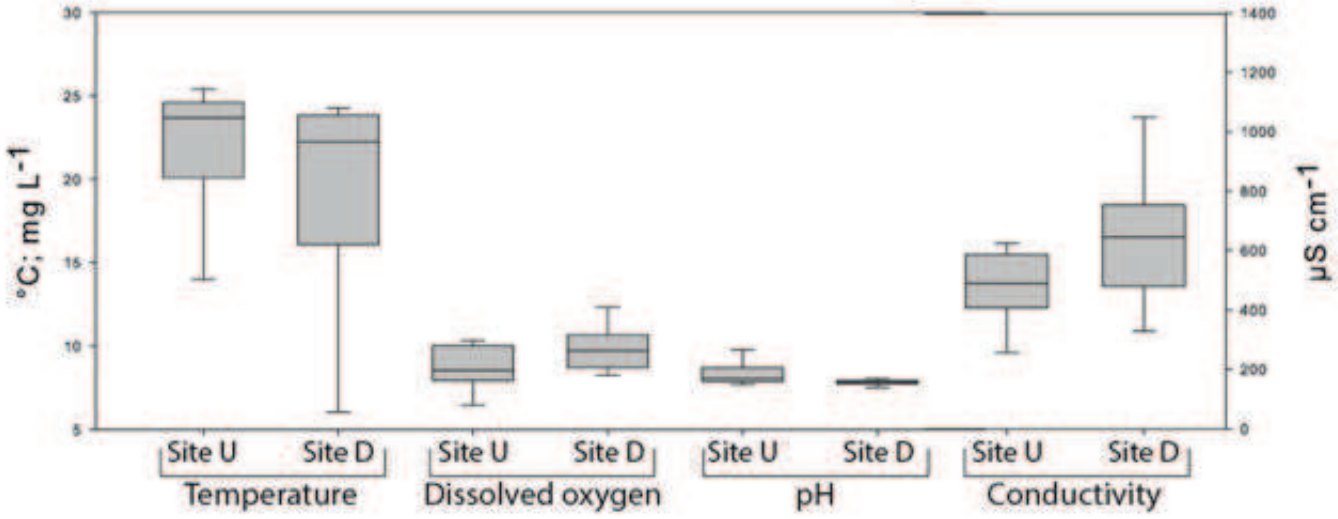

Figure 2 The boxplots rapresent chemical-physical variables measured in the two sampling points. Every boxplot show minimum, maximum, 5th and 95th percentiles, median.

Looking at the available chemical monitoring data collected by the local Environmental Agency (http://www.arpa.piemonte.it), Cr, chloroform and Ni were the only compounds that were frequently detected in the river but the data didn't show any statistically significant difference ($p > 0.05$, t-test) between the two sites (up- and downstream the discharge) (Figure 3). We can argue that, apart from conductivity, the river concentrations of chemical compounds, included those attributable to past productions, such as chromium, were not particularly influenced by the current plant discharge.

4.2 Monitoring of perfluorinated compounds

The fluoropolymer plant is still a very significant source of PFOA and short chain PFCA (PFPeA and PFHxA)

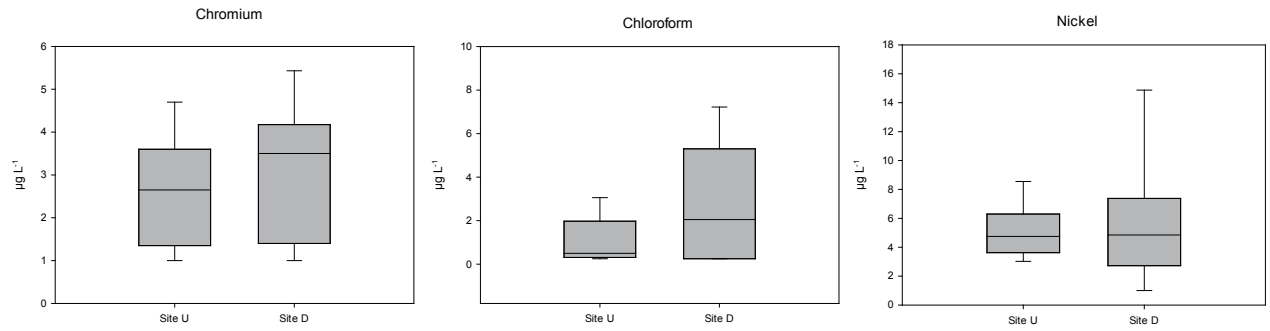

Figure 3 Boxplots illustrate the concentrations of Chromium ,Chloroform and Nickel measured in 2006 at the sampling sites. Boxplot show minimum, maximum, 5th and 95th percentiles, median.

for the river Bormida, as already identified in previous studies (Loos et al., 2008). A maximum PFOA concentrations of 6.5 µg L^{-1} was measured in the Bormida, downstream the fluorochemical plant, between July 2008 and July 2013, with concentrations ranging from 0.27 to 6.5 µg L^{-1} which were very variable because of the variability in river flow rates and industrial production cycles (Figure 5 and 6). Concentrations of most of PFAA in the upstream station were close to the detection limits, a part from PFHxA, PFHpA and PFOA which showed average concentrations of 2.0, 2.9 and 34.3 ng L^{-1} respectively. These concentrations could be attributed to some unidentified source in the upper part of the stream or to the atmospheric diffusion of PFAA from the chemical plant, as already demonstrated for PFOA in another fluoropolymer manufacturing facility site (Davis et al., 2007), or generated by atmospheric precursors such as 8:2 fluorotelomers (Prevedouros et al., 2006).

Observing concentration data in the period 2008-2013 (Figures 4 and 5), the use of PFOA in fluoropolymer production has not been decreasing and the annual average concentrations in the river Bormida ranged between 1.3 and 2.0 µg L-1 in this time range. Data collected in 2014 showed a decrease of PFOA concentration which might be attributed to a change in the production process, as claimed by the producer Company according to the Stewardship Program (US-EPA, 2006); nevertheless it should be confirmed by monitoring in the next years. Nevertheless the possible introduction of an alternative proprietary fluorinated surfactant in the polymerisation process, which is a functionalised polyfluoroalkyl ether carboxylic acids (C_3F_6ClO-$[CF_2$-$CF(CF_3)$-$O]_n$-$[CF(CF_3)$-$O]_m$-CF_2COOH, where n and m = 1; CAS 329238-24-6) already reported in the literature (Wang et al., 2013; European Food Safety Authority, 2010), is suggested by its detection in the Bormida waters by retrospective analysis using high resolution mass spectrometry (Mazzoni et al., in press)

On May 2012 we also analysed PFAA in sediments collected in both sites (Table 3). In the site downstream the plant we detected PFOA and higher chain compounds (PFDA, PFUnDA, PFDoDA) together with traces of PFHxS, while in the upstream site only few traces of PFOA and PFHxS have been determined. The PFOA

Table 3 PFAA concentrations (ng g^{-1}) in sediments (May 2012)

Sites	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFBS	PFHxS	PFOS
U	<LOD	<LOD	<LOD	0.3	<LOD	<LOD	<LOD	<LOD	<LOD	0.1	<LOD
D	<LOD	<LOD	<LOD	2.9	<LOD	0.4	1.1	0.7	<LOD	0.1	<LOD

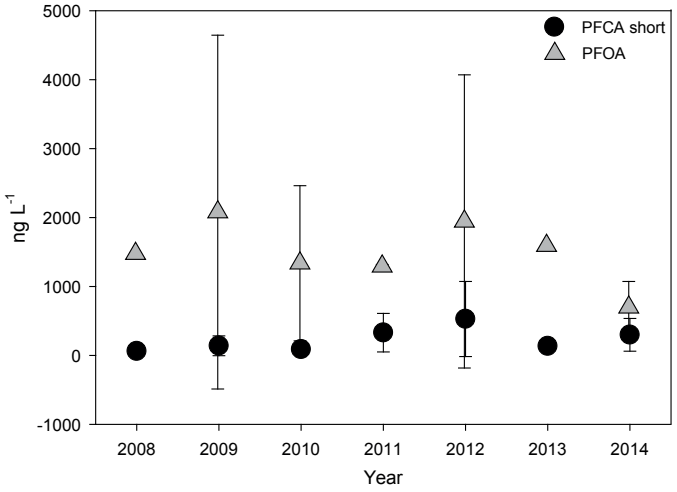

Figure 4 ShortPFCA and PFOA concentrations measured in the years (2008-2014)

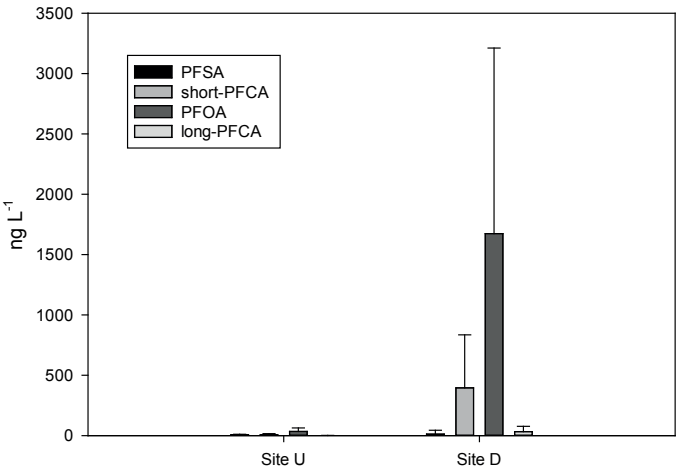

Figure 5 PFAA concentrations measured up- and downstram the industrial ischage in the years (2008-2014)

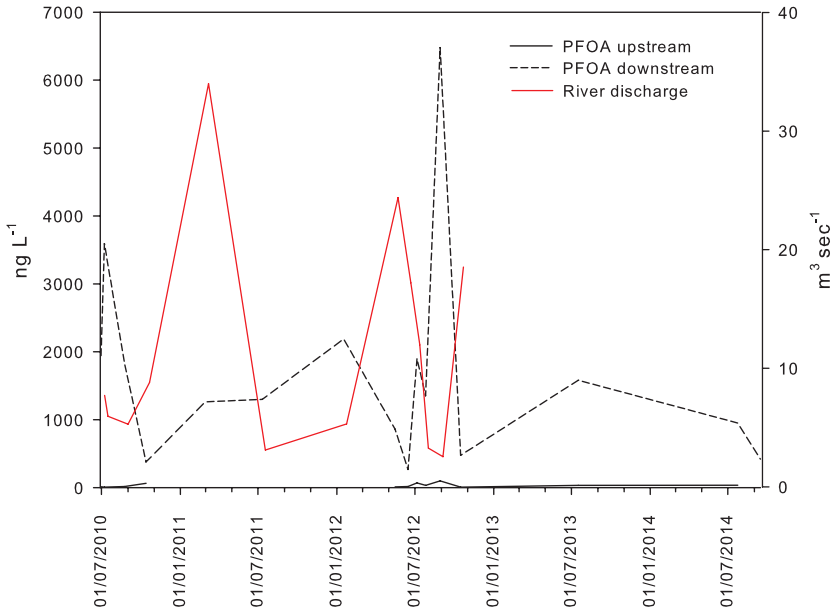

Figure 6 PFOA concentration and river discharge during the years

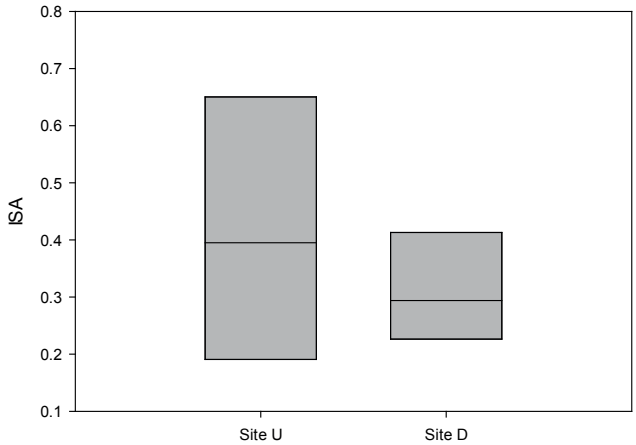

Figure 7 Artificial Substrate Index calculated for up- and downstream sites. Boxplot show 5th and 95th percentiles and median.

4.3 Ecological Quality

In the present study the sampling was performed by ASs to avoid problems in accessing the river in case of high flow and thereby ensure the sampling. The data have been evaluated by calculating the Artificial Substrate Index (ISA by MacrOper.ICM 1.0.5 software), but they did not show any difference in quality between sites U and D, as shown by box-and-whiskers in the Figure 7, although there was a greater variability in the upstream site in comparison with downstream one. Quality classification obtained in the present study was in accordance with the WFD ecological classification which was 'moderate' in the period 2009-2011 for both sites.

The analysis of the community was performed at lower taxonomic levels than family, at least at genus level and, when possible, at species level. This type of comparison was made because, inside the same family, genus or species may have different tolerance and resistance to specific pressures. Piecharts (Figure 8) show the percentage composition of taxa present in the two sites.

Upstream 73% of the community was composed of *Echinogammarus* (Gammarids, Crustaceans), 14% of chironomids, 4% of the genus *Choroterpes* mayflies, caddis, 3% of the family Hydropsychidae and 2% was mayflies *Caenis*. The remaining 4% was the sum of stoneflies, other mayflies, other Diptera and gastropods.

Downstream the community changed its structure: *Echinogammarus* constituted 88% of the total community, 7% chironomids, 2% Hydropsychidae and 1% *Caenis*. Downstream mayflies like Choroterpes disappeared and this suggests the presence of a stress in the intermediate section between the two sites that affects this sensitive component of the community. It can be highlighted that downstream the individual density was significantly higher: generally individuals collected downstream were twice those collected upstream and the abundance in June was almost 10 times.

A Principal Component Analysis (PCA, Figure 9 and 10) was performed using the data of abundance of taxa for each artificial substrate, for each season, for a total of 12 observations. In order to facilitate interpretation of the results, the first two principal components were correlated with environmental variables measured in each sample and the metrics calculated at the level of the family.

Eigenvalues related to the first two axes explain respectively 35.0% and 22.4% of the total variability and the samples are grouped according to site, regardless to sampling season.

Upstream samples are mainly characterized by the order of the mayfly taxa, in particular *Choroterpes* genus, while the downstream samples are characterized by the presence of gammarids, and Diptera (Simuliidae, Tipulidae, Anthomidae), Elminthidae and Hydropsychidae taxa. Along the second axis samples collected downstream in June are separated, characterized by very high density of gammarids such *Echinogammarus*. Site U was charachterized by the highest values of metrics like Shannon index, Sel _EPDT, number of EPT taxa, ASPT, while site D was characterized by a higher number of taxa and by higher values of dissolved oxygen. The chemical-physical variables, such as conductivity and pH, are very slightly correlated with the first two axes.

4.4 Genetic variability between populations

Twenty-four AFLP primer pairs were tested. Using three of the primer pairs we detected 181 polymorphic loci among the various samples of *H. modesta* (Table 4). Among the scored 181 fragments, 163 (90.05%) were polymorphic in individuals collected upstream and 150 (82.87%) in those collected downstream. Observed heterozygosity is reported in Table 5.

AMOVA analysis was carried out to test the presence of temporal shift in genetic variability within the studied stations. "June" populations were compared with "August" populations and no significant divergence was

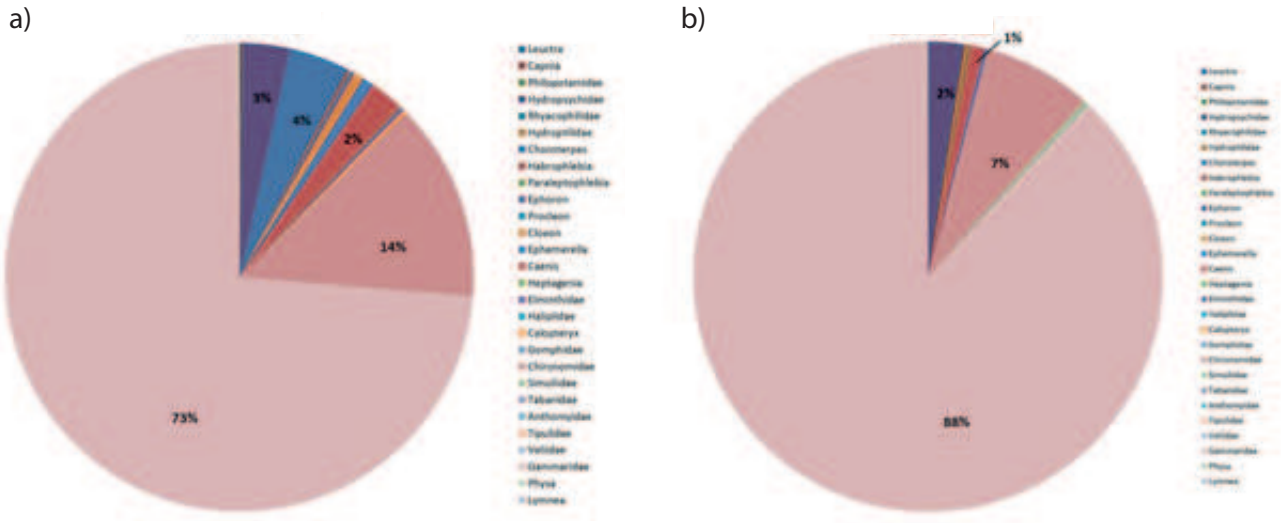

Figure 8 Community structure a) site U and b)site D

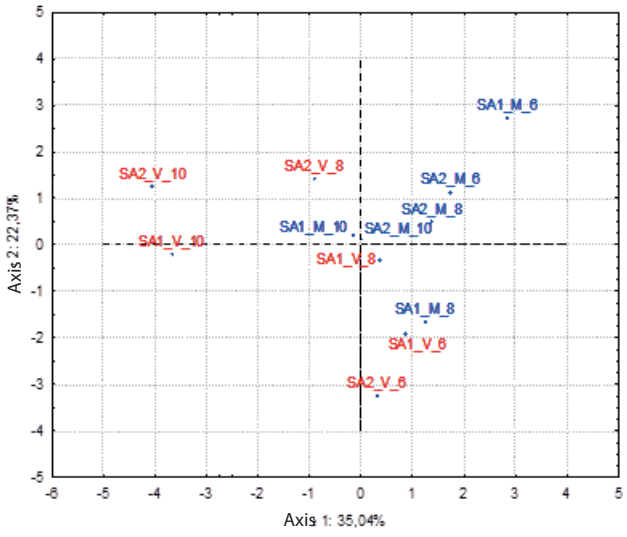

Figure 9 Score plot for the first two variables: SA = Artificial Substrate; M = Upstraem site; V = Downstream site; 6,8,10= sampling months (June, August, October)

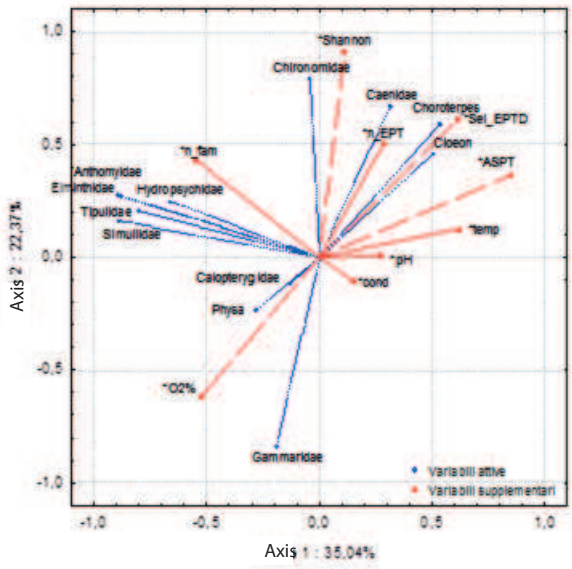

Figure 10 Loading plot for the first two variables

Table 4 Primers pairs selected

*Eco*RI primer	*Taq*I primer	Number of loci
E00	T32	57
E32	T49	52
E00	T49	72

Table 5 Observed heterozigosity

	Upstream population	Downstream population
Min	0.00000	0.00000
Max	0.52632	0.52632
Mean	0.27235	0.28238
s.d.	0.17765	0.18911

detected (Table 6).

In light of this evidence, we considered individuals collected upstream in the two sampling sessions as a unique population; the same procedure was applied to downstream samplings. Under these settings, AMOVA calculated a significant single index (F_{ST}) that is related to the variability between populations. The associated percentage of variation was 6.8% (F_{ST} =0.06791) (Table 7). Nevertheless, most of the variation was allocated at within population level.

Table 6 Results of AMOVA analysis testing "June" populations VS. "August" populations

SOURCE OF VARIATION	PERCENTAGE OF VARIATION	F-statistics	*P value*
Among groups	0.00	F_{CT} : 0.00009	0.34
Among populations within groups	8.15	F_{SC} : 0.08157	0.01
Within populations	91.85	F_{ST}: 0.08149	0.001

Table 7 Results of AMOVA analysis testing Upstream population VS. Downstream population

SOURCE OF VARIATION	PERCENTAGE OF VARIATION	F-statistics	*P value*
Among groups	6.79	FST : 0.06791	0.008
Within populations	93.21		

The MS-tree calculated on the basis of Euclidean distances is congruent with the indications provided by AMOVA, and does not represent a sharp distinction of upstream or downstream clades.

Nevertheless, six downstream haplotypes were gathered in a single subclade (Figure 11).

In any locus no significant ($p < 0.05$) differences, compatible with the presence of selective effects, were highlighted, as evident from the analysis performed by BayeScan software.

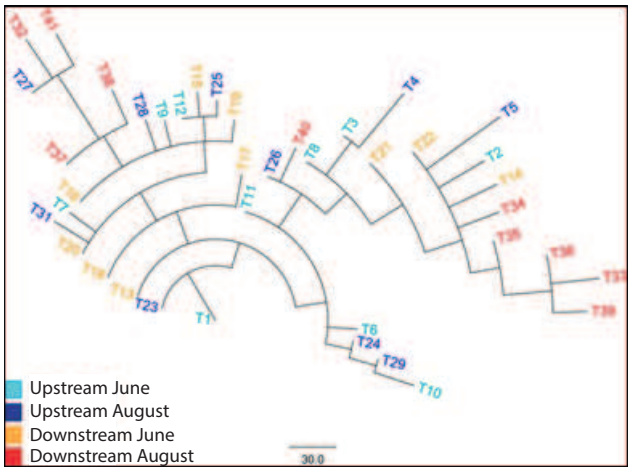

Figure 11 Minimum Spanning tree. T=Trichoptera

5. Discussion

Benthic communities, resident up- and downstream the point source of PFAS, were analyzed with different approaches in order to investigate whether the discharge pressure induce any variation in the community structure or effect at genetic scale on resident populations.

It is interesting to note that the PTFE production started in the '80 and thereby the ecological community has been exposed to a mixture of chemicals of industrial origin, which includes mainly poly- and perfluorinated compounds, since many decades. Among the analysed perfluorinated compounds, PFOA has been identified as the compound present at the highest concentrations in the river water, with an average concentration of 1.5 μg L^{-1} in the time range from 2008 to 2013 and a maximum concentration of 6.5 μg L^{-1}. These values are thousand times lower than the lowest No Observed Effect concentrations (NOEC) measured in chronic tests for freshwater invertebrates and crustaceans (Table 8). Nevertheless it is limitative to assess the risk of the community based only on the toxicity data of a limited list of monitored substances and effect-based assays have to be implemented.

The available toxicological data for the analysed compounds (Giesy et al., 2010; Hoke et al., 2012; Ding and Peijneburg, 2013) are in accordance with the experimental evidence that no clear effect on population can be evidenced neither as regard the community composition nor at the molecular level as a result of a selective pressure.

The community composition was slightly different in the two sampling points, with higher values of Shannon index, Sel_EPDT, number of EPT taxa and ASPT in U, confirming the presence of more sensitive taxa, and with the downstream site dominated by a larger number of taxa considered more resistant to pollution. Nonetheless, the overall quality of the sites, as calculated by the Environmental Agency according to the STAR-ICM multimetric index (calculated considering family level), did not show a difference between sites, which were both classified as moderate. It is remarkable that the Ephemeroptera genus *Choroterpes* is present only in the U site, being possibly affected by the industrial discharge. This proves that a finer taxonomical identification of specimens (to genus/species) may highlight important differences between sites, since genera/species belonging to the same family may show different responses to environmental pressures.

Table 8 PFOA: CHRONIC EFFECTS for freshwater (f) invertebrates and crustaceans

Species: Invertebrata - Crustacea		**Exp time**	**Endpoint**	**EC10 (95% CI) mg L^{-1}**	**NOEC mg L^{-1}**	**References**
Daphnia magna	f	21 d	growth (as length)		44.2	(Colombo et al., 2008)
Daphnia magna	f	21 d	reproduction		12.5	(Ji et al., 2008)
Moina macrocopa	f	7 d	reproduction		3.125	(Ji et al., 2008)
Daphnia magna	f	21 d	survival reproduction		>100 10	(Li, 2010)
Daphnia magna	f	21 d	reproduction growth(as length)		20 44.2	(OECD, 2006)
Daphnia magna	f	21 d 14 d 14 d	reproduction rate reproduction survival		22 8 60	(OECD, 2006)
Daphnia magna	f	21 d	survival reproduction	11.12 7.02		(Yang et al., 2014)

At lower level of biological organization, analysis of specimens belonging to the same species (*H. modesta*) present in both sites was carried out to assess potential impacts at population level.

Molecular analysis initially ruled out molecular variance linked to seasonality which allowed to consider all individuals collected as two separate samples to assess the spatial gradient respect to the discharge point. The variance percentage calculated by AMOVA suggested the existence of a selective pressure and the fact that the percentage was relatively low could be considered compatible with a selective pressure that does not act indiscriminately on the whole genome of the animal but only on targeted loci related to the type of stress, as demonstrated in other works (Paris et al., 2010, Williwms and Oleksiak, 2008; Bach and Dahllof, 2012). This evidence is consistent with the layout of the individuals in the MS-tree, where some downstream individuals, originating from the same node in the tree, assemble, identifying a trend that evolves separately from the rest of the population.

Nevertheless, BayeScan analysis showed no presence of outlier loci, so the hypothesis of selective pressure is not tested. The analysis, however, might find more statistical robustness increasing both the number of loci, to increase the probability to find selected loci, either increasing the number of populations upstream and downstream.

The alternative hypothesis is that the percentage of genetic variance pointed out among up- and downstream populations is related to an environmental factor like distance or river hydromorphology that created different environmental conditions. Similarly, the analysis of a greater number of populations collected along the longitudinal river axis could clarify the reciprocal role of isolation by distance, different hydromorphological features or contaminants to determine the onset of genetic differences found.

A very similar approach, based on AFLP, was used by Andre et al. (2010) to study the spatial pattern of genetic diversity in earthworms living in a Pb-mine site affected by different degrees of contamination. The authors found genetic diversity between populations related to the specific contamination. However the success may depend on the type of contaminant involved, which is certainly much more toxic respect the molecules considered in this study.

Evidently, the concentrations of the pollutants involved in this study did not determine significant genetic effects on the population of *H. modesta*. If such effects exist, they could be detected increasing the number of analysed loci, to increase the probability to run into loci under selective pressure, but also using a more specific technique: transcriptomic provides a snapshot of gene expression to provide information regarding developmental stage, life-history or responses in relation to particular environmental stressors. By this way it is easiest to evidence specific genetic differences which can be related to the pressure of the contaminant.

A laboratory study to investigate the evolutionary consequences of exposure to perfluoralkyl substances (PFOS, PFOA, PFBS) in a multigeneration toxicity test on *Chironomus riparius* was recently carried out (Stefani et al, 2014). Also in this work no significant effects were induced by exposure to PFOA, while an increased mutation rate have been determined for the other PFAS (PFOS and PFBS), but no selective effects.

6. Conclusions

The main aim of this work, unique for the type of investigated impact and for the integrated approach, was to assess whether the macrobenthic river populations exposed for long time to high concentrations of PFOA and other fluorinated compounds coming from an industrial point source can be subject to an impact that is not quantifiable with the traditional monitoring methods used for ecological classification under European

Table 9 PFAA concentrations in 2010-2014 samples collected in the 2 sampling sites.

	Sampling date	River discharge m^3 sec^{-1}	PFBA ng L^{-1}	PFPeA ng L^{-1}	PFHxA ng L^{-1}	PFHpA ng L^{-1}	PFOA ng L^{-1}	PFNA ng L^{-1}	PFDA ng L^{-1}	PFUnDA ng L^{-1}	PFDoDA ng L^{-1}	PFBS ng L^{-1}	PFHxS ng L^{-1}	PFOS ng L^{-1}
Site U	30/06/2010	7.7	n.d.	<LOD	<LOD	<LOD	13	<LOD	<LOD	<LOD	<LOD	<LOD	n.d.	7
	08/07/2010	6.0	n.d.	<LOD	<LOD	<LOD	9	<LOD	<LOD	<LOD	<LOD	<LOD	n.d.	7
	24/08/2010	5.3	n.d.	<LOD	2	<LOD	16	<LOD	<LOD	<LOD	<LOD	<LOD	n.d.	<LOD
	13/10/2010	8.8	n.d.	<LOD	15	27	64	<LOD	<LOD	<LOD	<LOD	<LOD	n.d.	14
	28/02/2011	34.0	n.d.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	11/07/2011	3.1	n.d.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	17/01/2012	5.3	n.d.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	16/05/2012	24.4	<LOD	<LOD	<LOD	<LOD	11	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	3
	15/06/2012	17.2	<LOD	<LOD	1	1	18	<LOD	<LOD	<LOD	1	<LOD	<LOD	9
	06/07/2012	12.0	<LOD	<LOD	2	3	70	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	7
	26/07/2012	3.3	<LOD	<LOD	2	1	33	<LOD	<LOD	<LOD	0.00	<LOD	<LOD	<LOD
	29/08/2012	2.5	<LOD	<LOD	1	2	100	<LOD	1	<LOD	1	<LOD	<LOD	15
	16/10/2012	18.5	<LOD	<LOD	<LOD	<LOD	8	<LOD	1	2	2	<LOD	<LOD	1
	17/07/2013	n.d.	<LOD	<LOD	<LOD	<LOD	34	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	7
	24/07/2014	n.d.	n.d.	<LOD	2	3	36	<LOD	<LOD	<LOD	<LOD	5	<LOD	3
	15/09/2014	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Site D	30/06/2010	7.7	n.d.	8	26	77	1945	4	<LOD	<LOD	<LOD	<LOD	n.d.	<LOD
	08/07/2010	6.0	n.d.	9	75	118	3592	10	<LOD	<LOD	<LOD	<LOD	n.d.	<LOD
	24/08/2010	5.3	n.d.	6	72	101	1828	18	12	6	<LOD	<LOD	n.d.	<LOD
	13/10/2010	8.8	n.d.	<LOD	23	32	378	<LOD	<LOD	<LOD	<LOD	<LOD	n.d.	1
	28/02/2011	34.0	n.d.	28	45	60	1262	30	8	5	<LOD	<LOD	n.d.	<LOD
	11/07/2011	3.1	n.d.	238	134	156	1300	19	<LOD	<LOD	<LOD	<LOD	n.d.	<LOD
	17/01/2012	5.3	<LOD	167	191	372	2189	70	32	36	19	17	<LOD	109
	16/05/2012	24.4	<LOD	<LOD	42	60	861	2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	15/06/2012	17.2	<LOD	<LOD	20	8	267	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	38
	06/07/2012	12.0	<LOD	585	186	131	1900	12	<LOD	<LOD	<LOD	11	<LOD	<LOD
	26/07/2012	3.3	205	256	77	65	1353	31	32	44	<LOD	<LOD	<LOD	<LOD
	29/08/2012	2.5	235	195	346	946	6480	38	22	11	1	3	<LOD	<LOD
	16/10/2012	18.5	173	<LOD	16	30	476	3	1	2	<LOD	<LOD	<LOD	<LOD
	17/07/2013	n.d.	85	23	62	50	1581	6	3	3	1	6	<LOD	5
	24/07/2014	n.d.	n.d.	<LOD	43	88	951	13	8	3	0	5	<LOD	<LOD
	15/09/2014	n.d.	340	28	26	72	420	13	5	1	1	3	<LOD	3

regulation. To achieve this goal we used a subcellular analysis that investigate the effects on genetic variability in dwelling populations. The data obtained allow us to assert that the discharge of the fluorinated plant did not disrupt both downstream communities and populations and did not differentiate them substantially from those residents upstream the pollution source. Our results suggest that the class of perfluorinated compounds may have a low chronic toxicity, or that compensatory factors at the community and ecosystem level (i.e. genic fluxes from not contaminated areas by flying adults or drift) can dilute eventual effects. The study could be considered propaedeutic to verify whether the genetic structure is really linked just to a spatial factor and if other taxa available in the sites present the same genetic structure observed for *H. modesta*.

The genetic study carried out in field confirms data obtained in previous laboratory studies which suggested that perfluorocarboxylic acids do not present a significant risk for the aquatic ecosystems at environmental concentrations (Hoke et al., 2010) and that the risk for some bioaccumulable PFAS is mainly related on the accumulation in the aquatic trophic chain which poses concern for the end predators and consumers, included humans.

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Conclusioni, ricadute e prospettive future

L' attività di questa tesi si è inserita in un programma di monitoraggio pluriennale dei PFAS nelle risorse idriche italiane che, iniziato nel 2008, è ancora in corso per valutare l'evoluzione della composizione della miscela di PFAS, in funzione dei cambiamenti negli impieghi produttivi, e per cercare di evidenziarne gli effetti sulle comunità biologiche anche mediante test di laboratorio.

L' analisi dei dati, effettuata nel corso di questa tesi, ha mostrato come in Italia negli ultimi quattro anni non vi sia stata una significativa modificazione delle emissioni di acidi perfluoroalchilici (PFAA), né in termini quantitativi né qualitativi, cioè nella composizione delle miscele emesse in ambiente.

Come risultato generale, si può concludere che il PFOA è il composto con le più alte concentrazioni e la maggior frequenza di rilevamento in tutti i tipi di acque. I dati di monitoraggio, che includono campioni prelevati in un sito a valle di un impianto di fluoropolimeri, mostrano che il PFOA è ancora largamente utilizzato nei processi produttivi ed è presente in prodotti di uso quotidiano. Inoltre non si osserva in modo evidente che il PFOA sia stato sostituito nei processi industriali nonostante i principali produttori abbiano sottoscritto lo "Stewardship Program" di US-EPA, l' agenzia di protezione ambientale statunitense (http://www.epa.gov/oppt/pfoa/pubs/stewardship/pfoastewardshipbasics.html), per la progressiva riduzione ed eliminazione del PFOA nei processi e nei prodotti finali entro il 2015.

Altri acidi perfluorocarbossilici (PFCA) frequentemente rilevati nelle acque superficiali sono PFHxA, PFHpA e PFNA. Il PFBA sembra essere significativamente aumentato negli ultimi due anni. Se si considera la distribuzione dei diversi PFCA in acque potabili e sotterranee si evince che la composizione è simile nelle due matrici anche se con una frequenza di rilevamento ridotta in acque sotterranee, mentre differenze sostanziali sono evidenti nella distribuzione di acidi perfluorosolfonici (PFSA) tra le diverse tipologie di acqua. Nelle acque di superficie, infatti, PFBS e PFOS vengono rilevati con frequenze e concentrazioni medie simili, mentre raramente si misura la presenza di PFHxS. Al contrario in potabili e sotterranee il PFOS ha concentrazioni medie e frequenza di rilevamento più alte, mentre PFHxS è stato rilevato con frequenza e concentrazioni simili a PFBS. Questo è attribuibile al fatto che l'inquinamento delle acque sotterranee riflette l'inquinamento di decenni scorsi quando erano più utilizzati PFSA a lunga catena, mentre attualmente viene fatto un più largo utilizzo di quelli con catena carboniosa più corta. Il PFBS è infatti il sostituto più utilizzato del PFOS che è stato sottoposto a restrizioni a norma della Direttiva 2006/122/CE.

Inoltre, i risultati dell'indagine in materia di emissioni PFAS da diverse attività antropiche (discusso in due articoli pubblicati da Castiglioni et al., 2015 e Valsecchi et al., 2014 inclusi nella presente tesi) ci permettono di raggiungere le seguenti conclusioni:

- *Emissioni urbane.* PFAA sono presenti in molti prodotti di consumo e di uso quotidiano e quindi gli agglomerati urbani possono essere considerati sorgenti puntuali (mediante gli scarichi degli impianti di depurazione) e anche diffuse (dal run-off) di questi composti per i corpi idrici recettori. Sono stati calcolati i fattori di emissione urbani (EF) che corrispondono a 10 μg/die pro capite per il totale degli PFAA e 5 μg/die pro capite per PFOA, stimati sui valori medi settimanali in uscita da tre diversi impianti di depurazione di Milano, caratterizzati da una bassa componente industriale (<4%) (Castiglioni et al., 2015). Questi EF sono inferiori a quelli stimati dalle concentrazioni fluviali nei paesi industrializzati (Pistocchi

e Loos, 2009), ma molto vicino a EF misurati in impianti di depurazione di scarichi urbani coreani (3,5 µg/die pro capite per PFOA (Kim, 2012)).

- *Impianti industriali di fluorocomposti e fluoropolimeri.* Nei bacini studiati abbiamo identificato due impianti che producono intermedi di fluorocomposti e fluoropolimeri. L'impianto di fluoropolimeri, situato in Piemonte sul fiume Bormida, un affluente del fiume Tanaro nel bacino del Po, è ancora la principale fonte di PFOA e PFCA a corta catena (PFPeA e PFHxA) come già identificato in passato (Loos et al., 2008), con concentrazioni massime di PFOA, fino a 6,5 µg/L, misurate nel fiume Bormida. Tra il 2008 e il 2013 non è stata rilevata nessuna tendenza di riduzione nell'uso del PFOA per la produzione di fluoropolimeri. Il secondo importante impianto si trova a Trissino, provincia di Vicenza, regione Veneto, che produce molecole fluorurate, come PFOA e PFBS. L'impianto scarica i propri reflui nell'impianto di depurazione comunale la cui uscita è poi collettata con quelle di altri quattro impianti di depurazione in un unico collettore (chiamato Collettore ARICA) che recapita i suoi reflui nel fiume Fratta-Gorzone, affluente a sua volta del fiume Brenta, a valle della zona di ricarica delle falde vicentine. Se si considera le composizioni in percentuale dei PFAA nell'acqua del fiume Fratta-Gorzone e negli effluenti recapitati dal Collettore ARICA, queste sono molto simili alla composizione degli effluenti scaricati dall'impianto fluorochimico nel quale i principali composti sono PFBS (68%), PFHxA (11%), PFOA (10%) e PFPeA (8%). La costante presenza ad alte concentrazioni di PFBS è attribuibile al fatto che questa molecola sostituisce nelle sue applicazioni industriali il PFOS soggetto a restrizioni comunitarie ed incluso recentemente nella lista dei POP (Persistent Organic Pollutant) di Stoccolma e nella lista delle sostanze prioritarie secondo la Direttiva 2013/39/UE.

- *Uso di PFAA in applicazioni industriali.* Il bacino del fiume Arno presenta una situazione ideale per distinguere l'impatto di attività tessili e conciarie. Qui, infatti, i due distretti industriali sono vicini ma ben separati ed è stato possibile identificare il PFOA e suoi omologhi a catena più corta (soprattutto PFHxA e PFDA) come principali molecole derivanti dalle attività tessili dell'area di Prato, mentre dalle attività conciarie dell'area di Pisa deriva un impatto significativo di PFSA e in particolare di PFBS.

In conclusione, i dati raccolti in questa indagine permettono di evidenziare la presenza diffusa di PFOA, il composto più frequentemente rilevato in tutti i tipi di acque italiane e presente con le più alte concentrazioni nonostante le misure sostitutive e programmi di sostituzione ed eliminazione lanciati da produttori e utilizzatori di PFAA. Si è rilevata la crescente presenza di composti a corta catena (PFHxA e PFBS in particolare) a seguito della sostituzione degli omologhi a lunga catena (rispettivamente PFOA e PFOS) da parte dei produttori. Inoltre, solo da un'analisi retrospettiva preliminare, sono stati individuati nuovi composti fluorurati meno indagati, mostrando la necessità di ulteriori programmi di monitoraggio dei composti poli- e perfluorurati usati come sostituti per i PFAA (e non appartenenti alla classe PFAA).

I dati di monitoraggio raccolti in questa indagine italiana sono stati raccolti in un database georeferenziato che verrà reso accessibile al pubblico mediante il collegamento al portale IP-Chem, piattaforma d'informazione per il sito di monitoraggio chimico (http://ipchem.jrc.ec.europa.eu/) gestito dal Joint Research Centre di Ispra (VA) della Commissione Europea. I dati sono stati già forniti al Joint Research Centre di Ispra per essere inseriti nel database utile al processo di prioritizzazione delle sostanze pericolose per la revisione della lista delle sostanze prioritarie nelle acque nell'ambito della Direttiva Quadro sulle Acque (WFD). Gli stessi dati sono stati inoltre condivisi con la comunità scientifica che studia gli inquinanti emergenti mediante l'inserimento degli stessi nel database europeo EMPODAT del Network NORMAN (Network of reference laboratories, research

centres and related organisations for monitoring of emerging environmental substances) (http://www.norman-network.net/empodat/).
Tra le attività in corso c'è anche la derivazione di standard di qualità ambientale per un numero selezionato di PFAA (PFBA, PFPeA, PFHxA, PFOA e PFBS) per conto del Ministero dell'Ambiente italiano al fine di aggiungerli alla lista degli inquinanti specifici, definiti a livello nazionale, che dovrà essere aggiornata nella revisione del decreto italiano DM 260/2010 sulla classificazione dei corpi idrici superficiali.
Allo stato attuale gli standard di qualità sono derivati sulla base della letteratura disponibile che risulta piuttosto limitata soprattutto per quelle molecole a catena più corta inserite nelle produzioni solo recentemente. Per avere un quadro più completo e degli standard ambientali più accurati, sarebbe sicuramente utile disporre di molti più dati derivanti da studi tossicologici a tutti i livelli della catena trofica. Per questa finalità risultano sicuramente utili i test multigenerazionali di laboratorio che si pongono come efficiente metodologia per rilevare effetti cronici che agiscono a livello subcellulare e che si trasmettono di generazione in generazione (Ahrens and Bundschuh, 2014; Stefani et al., 2014). I dati di letteratura finora disponibili danno indicazioni di una bassa tossicità cronica sia di PFOA sia di altri PFAA anche a livello genetico. Questa evidenza giustifica che i valori di PNEC (Predicted No Effect Concentration) o gli EQS siano molto più elevati dei livelli ambientali misurati. Tuttavia il rischio per le molecole che bioaccumulano sembra riguardare i predatori terminali della catena trofica e il consumatore umano, per gli elevati tempi di emivita negli organismi superiori, specie per i mammiferi, nei quali sono stati misurati anche effetti di alterazione endocrina.

Il contributo del lavoro di ricerca svolto in questi anni ha colmato profonde lacune di conoscenza sulla situazione italiana e ha permesso di ottemperare alle richieste della WFD che prevede programmi di monitoraggio calibrati sulle situazioni locali. Sebbene a questo punto dell'indagine si sia ottenuto un quadro esauriente per buona parte dei bacini, esistono ancora delle realtà da indagare per giungere alla definitiva identificazione delle sorgenti sul territorio italiano. E' inoltre fondamentale proseguire nella conoscenza dei meccanismi di azione di queste sostanze negli organismi acquatici in modo da ottenere una valutazione del rischio affidabile per queste sostanze e sviluppare metodologie di indagine e monitoraggio basate sulla misura di effetti biologici da utilizzare in campo in particolare in siti soggetti a particolari pressioni urbane o industriali.

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