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Monitoring of pharmaceuticals in aquatic biota (*Procambarus clarkii*) of the Doñana National Park (Spain)

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ABSTRACT

In this work the presence of different pharmaceuticals at Doñana National Park (Spain) and their main entry sources (input source or entry points) have been stated over the 2011–2016 years period. Twenty-three selected pharmaceuticals (corresponding to eight therapeutic families) were evaluated in crayfish and water samples from Doñana National Park (Spain) (six sampling points selected in order to cover different possible pollution sources into and surrounding the Park). The multiresidue determination was carried out using enzymatic-microwave assisted extraction prior to high performance liquid chromatography mass spectrometry detection. Sulphonamides (sulfadiazine, sulfamerazine, sulfamethazine, and sulfamethoxazole); trimethoprim, an antibiotic that is frequently co-administered with sulfamethoxazole; amphenicols (chloramphenicol, florfenicol and thiamphenicol); fluoroquinolones (ciprofloxacin, enrofloxacin, flumequine, danofloxacin, gatifloxacin, norfloxacin, marbofloxacin and grepafloxacin); penicillins (amoxicillin); tetracyclines (chlortetracycline and oxytetracycline); non-steroidal anti-inflammatory drugs (salicylic acid and ibuprofen); beta-blocker drugs (atenolol); and anti-epileptics (carbamazepine) were analysed. Ciprofloxacin, ibuprofen, salicylic acid, flumequine, and carbamazepine were detected and/or quantified at some of the selected sampling points. A clear ecotoxicological risk to the ecosystem was demonstrated from the occurrence of ciprofloxacin in samples obtained after the punctual and massive presence of people inside the Park. Furthermore, flumequine and carbamazepine have been detected in *Procambarus clarkii* specimens in concentrations around 30 ng g^{-1} and 14 ng g^{-1}, respectively, and their occurrence in the specimens could indicate the persistence of the discharge sources. The main source of pharmaceuticals into the Park might be the livestock farming activities, and the influence of urban wastewaters from surrounding villages does not seem to be very important.

1. Introduction

Conventional pollutants, such as metals or pesticides, have for many years been subjected to strict use and discharge controls and most of them are routinely monitored. This fact has usually led to a decrease on their levels in the areas of influence of pollution sources. However, pollutants of emerging concern (PECs) have not been currently considered and consequently, their monitoring and adverse effects on the environment, alone or in association with conventional pollutants, have gone largely unnoticed. Numerous studies have demonstrated the penetration of these pollutants into apparently pollution-free areas causing in some cases severe effects including development of resistant bacterial populations and exposure with potential drug accumulation in aquatic fauna and flora (Rigos et al., 2004; Chaturvedi et al., 2021).

PECs have been broadly defined as chemical substances, or even microorganisms, that traditionally are not monitored in the environment in contrast to classical pollutants, nevertheless, its long-term continuous release has been widely demonstrated (Molina et al., 2020; González-Mira et al., 2018). Their persistence in the environment can result in biota and humans exposure, however, many of these pollutants have not been detected until the sensitivity of analytical methods were improved. Additionally, the use of new chemicals or changes in the use of existing ones must be considered as new sources of their release into the environment.

Pharmaceuticals, and their metabolites and degradation products, are a well-known group of PECs. Their continuous discharge and

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pseudo-persistence in the environment as well as their potential ecotoxicological effect suggest the ecosystem and effluent monitoring need (Rodil et al., 2012; Chaturvedi et al., 2021). Except for the influence of effluents from pharmaceutical industries in some places, the main sources of these pollutants are urban wastewaters discharges (including those undergoing treatment) and effluents from aquaculture and livestock activities (Vystavna et al., 2012; Bendz et al., 2005; Comeau et al., 2008). However, the presence of pharmaceuticals, generally, is not only restricted to the aquatic environment close to the above-mentioned sources; but also surface and groundwater streams can act as vectors for the spread of these pollutants and they can be found at significant distances from primary discharges (Ferrando-Climent et al., 2014; Kandie et al., 2020; Szymczycha et al., 2020; Li et al., 2020).

Several studies have been published, so far, dealing with the occurrence of pharmaceuticals in environmental waters (Scott et al., 2014; Rodríguez-Mozaz et al., 2016; Osorio et al., 2016; Rogowska et al., 2020; Kalka et al., 2020; Majumber et al., 2019) as well as many focused on sediments (Vazquez-Roig et al., 2012; Llompart et al., 2019) and soils (Kodešová et al., 2019; Golovko et al., 2016). Although to a lessen extent, some studies have attempted to assess the risk in the aquatic environment by the analysis of pharmaceuticals in environmental biota (Aceña et al., 2016; Miller et al., 2018, 2019; Huerta et al., 2018; Alvarez-Muñoz et al., 2015; Moreno-González et al., 2016; Boillot et al., 2015; Dodder et al., 2014; Rösch et al., 2016). It is therefore clear that the determination of pollutants in a biota ecosystem provides more information and helps to understand the cause-effect relationships regarding their environmental impact than the measurement of discrete levels on water, soil or air samples. Furthermore, ecological and biochemical studies of the exposed biota allow its use as an environmental or ecosystem health indicator (bioindicators). Reports focused on the evaluation of the environmental pollution from different sources in the surroundings of the Doñana national park (DNP) (Garrido et al., 2016; Barón et al., 2014, 2015) have been published. The results evidenced that both, agricultural activities and discharges from wastewater treatment plants (WWTPs) in highly populated cities negatively affect the wildlife sanctuary. On the other hand, studies on free-living *Mus spretus* mice, used as bioindicators, have assessed the pollution status of areas surrounding DNP through iTRAQ (isobaric tags for relative and absolute quantification) analysis of their hepatic proteins (Abril et al., 2015).

There are limited studies in diverse zones of the park and surrounding areas, and only some papers related to measuring priority pollutants have been published. Therefore, there are some data on levels of pharmaceuticals in surface waters within the park. Thus, levels of some pharmaceuticals from environmental waters and urban wastewater treatment plants effluents were reported (Camacho-Muñoz et al., 2010a,b), likewise, some pharmaceutically active compounds were measured in sediments from DNP and surrounding areas (Camacho-Muñoz et al., 2013).

In the 70s of the last century, the american red swamp crayfish (*Procambarus clarkii*) was introduced in the surroundings of DNP with the aim of its exploitation as a fishery resource. Acclimatization of the specie was a success and soon started to be exploited commercially. The economic benefit reported and the ease of finding live crabs on the market encouraged their exploitation in areas with similar habitats to repeat the experience. Climatic conditions on the south of the Iberian Peninsula and the absence of natural predators allow to a wide and fast expansion of this crayfish specie and, nowadays, *Procamburus clarkii* is spread throughout practically all the Iberian Peninsula and several zones of Europe. *P. clarkii* was also a carrier of a disease caused by the fungus *Aphanomyces astaci*, which had a devastating effect on European crayfish (*Austropotamobius pallipes*) populations, in addition to their negative effect on the agricultural infrastructures due to digging galleries in the areas it occupies. The native crayfish, already hard hit by the loss of water quality of the rivers they inhabited, was decimated by this plague and today *A. pallipes* only survives in the headwaters of some clean, cold rivers inaccessible to *P. clarkii*. Given its presence throughout DNP and its physiological tolerance of low levels of dissolved O_2 (2–14 mg L^{-1}), changes in temperature (17-22 °C) and high rates of pollution, *P. clarkii* might be an appropriate environmental bioindicator specie. Furthermore, the presence of *P. clarkii* in the park makes it possible to spread the contamination effect to other species.

Some papers have been published using *P. clarkii* as bioindicator by correlating some biochemical parameters and environmental pollution (Vioque-Fernández et al., 2007, 2009; Osuna-Jiménez et al., 2014; Fernández-Cisnal et al., 2014).

In this work, specimens of *P. clarkii* were sampled within the years 2011–2016 from six zones of the DNP which were selected in order to cover different pollution sources into and surrounding the Park. Additionally, water samples from the same sampling points were also collected and analysed. Crayfish specimens were analysed according to a previously described analytical procedure (Kazakova et al., 2018) and 23 pharmaceutical active principles corresponding to 8 therapeutic families were analysed: sulphonamides (sulfadiazine, sulfamerazine, sulfamethazine, and sulfamethoxazole); trimethoprim, an antibiotic that is frequently co-administered with sulfamethoxazole; amphenicols (chloramphenicol, florfenicol and thiamphenicol); fluoroquinolones (ciprofloxacin, enrofloxacin, flumequine, danofloxacin, gatifloxacin, norfloxacin, marbofloxacin and grepafloxacin); penicillins (amoxicillin); tetracyclines (chlortetracycline and oxytetracycline); non-steroidal anti-inflammatory drugs (salicylic acid and ibuprofen); beta-blocker drugs (atenolol); and antiepileptics (carbamazepine).

The main goal of this work is exploring the use of specimens of *P. clarkii* as possible bioindicators of the environmental pressure for pharmaceuticals that the Doñana National Park could suffer in the aquatic media. Additionally, the identification of possible pollution sources will be discussed.

2. Materials and methods

2.1. Chemicals and standards

Sulfadiazine (SDZ), sulfamerazine (SMR), sulfamethazine (SMT), and sulfamethoxazole (SMX), trimethoprim (TMP), chloramphenicol (CLF), florfenicol (FLF), thiamphenicol(TIF), ciprofloxacin (CPR), enrofloxacin (ENR), flumequine (FMQ), danofloxacin (DNF), gatifloxacin (GTF), norfloxacin (NRF), marbofloxacin (MRB), grepafloxacin (GPF), amoxicillin (AMX), chlortetracycline (CLT), oxytetracycline (OXT), salicylic acid (SAC) ibuprofen (IBU), atenolol (ATN) and carbamazepine (CBZ) (97–99.9% purity) were purchased from Fluka-Sigma-Aldrich, S.A. (Madrid, Spain). *Proteinase-K* (20.2 mg mL^{-1}) recombinant PCR grade was supplied from Roche Diagnosis (Barcelona, Spain). Acetonitrile and methanol (LC-MS grade) and formic acid (analytical grade) were purchased from VWR (Barcelona, Spain). Ultrapure deionized water (Milli-Q Plus water system Millipore (Billerica, MA, USA)) was used for all aqueous solutions and dilutions.

Stock solutions were prepared by dissolving the substances in methanol, except CLF, FLF, OXT, CLT and TIF which were dissolved in ultrapure water and CPR by a 80:20 v/v methanol:water mixture. 400 mg L^{-1} stock solutions were prepared for all active pharmaceutical substances, except for CLT, OXT and CPR which 100 mg L^{-1} ones were prepared. All the stock solutions were stable for at least 2 months at 4 °C, except CPR, CLT and OXT solutions that must be prepared daily and kept at room temperature due to the low solubility of these substances. Working solutions were daily prepared by adequate dilutions using ultrapure water.

2.2. Sampling plan in Doñana National Park

DNP is a protected area over 100.000 ha and it is located in the Guadalquivir estuary in Andalusia (Spain). Within this area, it is the Doñana Natural Reserve with 54.252 ha, which is integrated in the

Ramsar Convention for the protection of wetlands and recognized by UNESCO, first as a Biosphere Reserve and then as a World Heritage Site. DNP has a biodiversity that is unique in Europe.

A sampling plan (from 2011 to 2016) for *P. clarkii* specimens and surface water samples collection was designed in order to select the sampling points and the most appropriate seasons. Fig. 1 shows the location of the selected sampling points, and their corresponding UTM coordinates. Briefly, the main characteristics that determined the sampling areas from an environmental point of view are also described below. These sampling points were selected in order to determine potential impacts from emerging pollutants and, essentially, considering the possible environmental pressure from emerging pollutants such as the selected ones. Also, it must be considered that the water streams that cross the DNP receive the effluents of wastewater treatment plants (WWTPs) of some villages.

Rocina (ROC) corresponds to a sampling point in the headwaters of the stream "La Rocina". This site could be influenced by livestock husbandry and could be also affected by the intensive strawberries plantations in greenhouses located in the sourroundings. The sampling point is close to the village of "El Rocío", with a relatively low stable population which significantly increases during the weekends, and it should be noted that for one week a year, its population grows dramatically exceeding a million inhabitants due to a popular annual pilgrimage. The effect of the punctual high population on the levels of emerging pollutants has been verified according to the results obtained.

Bernabé (BER) is also a sampling point located in the lower watershed of the stream "La Rocina" but near of "El Rocío", and the influence of livestock husbandry and citrus crops must also be considered from an environmental point of view.

Partido (PAR) corresponds to a sampling point in the headwaters of the stream "El Partido". Citrus crops and the presence of horses must be considered as water pollution sources.

The sampling point Ajolí (AJO) is also located in the stream "El Partido" but near of the village "El Rocío". On this point, besides the influence of citrus crops, it is necessary to consider the presence of horse and cattle farming activities.

Matochal (MAT) is a sampling point located near the Guadiamar river and the external western limits of the Park. The presence of pollutants in this zone can be conditioned by the nearby and widespread rice crops, the Aznalcollar mines in the headwaters of the Guadiamar river (infamous for an ecological mining disaster in 1998) and urban wastewater discharges from the villages: Aznalcazar, Pilas and Isla Mayor. The influence of water from the Guadalquivir river must be also considered.

Lucio del Palacio (LPD) was initially considered as "clean sampling point" due to its location in the innermost part of the Park, and not influenced by external pollution sources. However, the close presence of Doñana's Singular Scientific-Technical Infrastructure (ICTS-RBD) for DPN personnel and researchers could condition some environmental pressure.

The sampling period was also determined taking into account the abundance of the *P. clarkii* species according to the climate. Climatological data were obtained from two stations located in the surroundings of the DNP: RAIFSE004 "Phytosanitary Warning and Information Network (CAPDR)" (Red de Alerta e Información Fitosanitaria, Consejería de Agricultura y Pesca, Junta de Andalucía, Spain) and the station RIA2110 "AgroClimatic Information Network (IFAPA)" (Red de Información AgroClimática (IFAPA), Instituto de Investigación y Formación Agraria y Pesquera, Junta de Andalucía, Spain). Table S1 and Table S2 (supplementary electronic data) provide data on average recorded monthly temperature and average monthly rainfall over the period 2011–2016. DNP has a mild, typically Mediterranean climate with Atlantic influence, rainfall tends to be scarce in summer and relatively generous in winter; however, rainfall is quite variable. The average rainfall (580 mm) is mainly usually distributed from September to April. Thermic oscillation between maximum temperatures throughout the year is about 17 °C. From the end of spring to the end summer, the presence of a subtropical anticyclone located near Azores Islands leads to a dry and quite hot period. However, certain zones of the Park retain water streams due to external contributions from rivers and rivulets. Both, temperature and rainfall affect the crayfish abundance and the dilution or concentration of possible pollutants in the water streams. For this reason, spring (from May to June) was the most appropriate season.

2.3. Sample collection, preparation and extraction

P. clarkii specimens and surface water samples were collected in the period from May to June 2011 to 2016 at the six sampling points (one time/24 h sampling) located in the Natural Reserve of Doñana described in previous section and its surrounding areas.

The number of specimens captured each year was variable due to hydro-meteorological conditions; however, an average number of approximately 100 specimens per year in the set of locations were collected using traps placed in waterways and collected after 24 h. Captured *P. clarkii* were shipped under cool conditions (<4 °C) to our laboratory. Muscle tissue without ventral nerve cord and the hepatopancreas (viscera) from each specimen were obtained for subsequent analysis and they were stored for less than 5 day at −80 °C before analysis. Frozen samples were lyophilized and then homogenized using a grinder to obtain two sets of sample pools from each sampling point. Samples were kept at −80 °C until their extraction and analysis.

P. clarkii samples were extracted according to a previously optimized extraction procedure (Kazakova et al., 2018). Thus, 0.5 g of each sample pool of lyophilized muscle or viscera samples was placed into pressurized PTFE microwave vessels and then a mixture containing 10 mL of acetonitrile:water (1:1, v/v), 50 μL of commercial *Proteinase-K* solution and 5 μL of concentrated formic acid was added. 50 W microwave power (Ethos One, Millestone, Sorisole (BG), Italy) was applied for 5 min. Cooled (room temperature) muscle and viscera samples were centrifuged at 7000 rpm for 10 min and 20 min, respectively. First, the liquid extracts were evaporated under a nitrogen stream, then dissolved using 1 mL of 0.1% (v/v) aqueous formic acid solution and filtered through a 0.22 μm nylon syringe filter. Finally, a 10 μL aliquot was directly injected into the chromatographic system. For viscera samples, an additional centrifugation step (20 min at 7000 rpm) was applied previous to microfiltration.

Additionally, water samples from each sampling point were also collected using precleaned polypropylene (PP) bottles. The collected samples were shipped at temperature < 4 °C. Water samples were filtered through a nylon 0.45 μm filter prior extraction. Extraction was carried out, with slight modifications, according to the proposed procedure of Gros et al. (2009). For SPE extraction, a 12-port Visiprep™ Standard vacuum manifold (Supelco, Sigma-Aldrick, Madrid, Spain) was used. Oasis HLB cartridges (60 mg) (Waters Corporation, Milford, MA, USA) were previously conditioned using 5 mL of methanol and 5 mL of analytical-grade water. 500 mL of filtered water were passed through the SPE cartridge at a flow rate of 4–5 mL min^{-1}. The cartridges were then washed with 5 mL of water and they were vacuum-dried for 15 min. Elution was carried out using 2 × 4 mL of methanol and the extracts were evaporated to dryness under nitrogen stream. The residues were reconstituted in 1 mL of analytical-grade water and filtered (0.22 μm) prior injection for its analysis (10 μL).

2.4. Instrumental analysis

Analysis of extracted muscle, viscera and water samples were performed using a previous procedure (Kazakova et al., 2018). Briefly, an Agilent 1290 Infinity UPLC system was used and the separation was achieved using a reversed-phase Zorbax Eclipse XDB-C18 analytical column (150 mm × 4.6 mm, particle size 3 μm) preceded by a guard-column Zorbax ODS (4.6 mm × 12.5 mm, particle size 5 μm)

Area (code) (location)	UTM coordinates	
	X	Y
Rocina (ROC) (upstream La Rocina stream)	178,653	4,119,937
Bernabé (BER) (La Rocina stream, near "El Rocio" village)	187,036	4,116,086
El Partido (PAR) (upstream El Partido stream)	191,173	4,124,977
Ajoli (AJO) (Partido stream, near "El Rocio" village)	192,352	4,115,565
Matochal (MAT) (Guadiamar stream, near Doñana National Park)	208,681	4,102,207
Lucio del Palacio (LDP) (inside Biological Reserve of Doñana)	193,800	4,099,515

Fig. 1. Location of the sampling points.

(Agilent Technologies Spain S.L). Binary gradient elution programs at 0.4 mL min^{-1} flow rate were performed using 0.1% (v/v) formic acid aqueous solution (A) and acetonitrile (B) as mobile phase. For positive ionization mode, the gradient was: (1) 12 min linear elution gradient from 0% to 30% B, (2) 5 min linear gradient to 100% B and (3) 4 min of isocratic step. For negative ionization mode, the used gradient was: (1) 15 min linear elution gradient from 10% to 30% B and (2) 6 min linear gradient to 100% B. In both cases, the composition was returned to initial conditions (0% B and10% B for positive and negative ionization mode, respectively) and 5 min were waited before the next injection.

Detection was performed by an Applied Biosystems 3200 QTRAP LC/MS-MS instrument (ABSciex, Foster City, USA), an hybrid triple quadrupole linear ion trap (QqQLIT) mass spectrometer. Ionization was performed by an electrospray ion source (TurboSpray®). Multiple Reaction Monitoring (MRM) mode was used for the chromatographic detection, selecting $[M+H]^+$ and $[M\text{-}H]^-$ as precursor ions for positive and negative ionization mode, respectively. Other mass spectrometer parameters were: (1) collision gas (nitrogen) at 4 psi, (2) ion source and curtain gases at 30 psi (positive ionization) and 20 psi (negative ionization),(3) electrospray (TurboSpray®) voltages: 5.5 kV (positive) and −4.5 kV (negative), (4) source temperature 500 °C for both modes. Two analytical signal were used: the most intense for quantification and the second one for qualitative confirmation.

3. Results and discussion

Section 2.2 describes the sampling plan for crayfish specimens and water samples from DNP and surroundings in order to obtain representative samples of the diverse environmental situations in the area related to the possible sources of pharmaceuticals contributions to the DNP aquatic media. An excellent report on the hydrology of the DNP and surrounding areas have been published (WWF World Wildlife Fund España, 2009).

Fig. 2 shows the hydrological functioning scheme for the studied area and the locations of the urban wastewater treatment plants around the DNP. As can be seen, DNP receives surface waters contributions from Guadalquivir and rivers and some minor water streams. Therefore, surface waters that contain discharges from urban WWTPs penetrate to deep areas of the Park. Additionally, despite the fact that the Guadalquivir river receives effluents from a significant number of WWTPs throughout its entire route, its low flow into the park as well as the influence of tidal waters means that its influence on the aquatic media of the park should be considered scarce.

The presence of pharmaceuticals in effluents from urban wastewater treatment plants (WWTPs) located in the surroundings of DNP have been previously described (Camacho-Muñoz et al., 2010a; Camacho-Muñoz et al., 2010b) so a priori it would be possible to think that this contamination could reach deep areas of the park.

According to the analytical protocols described in the experimental section, Table 1 shows the obtained results for the analysis of water and *P. clarkii* collected samples. Analytical details for the applied method (detection and cuantitation limits, chromatograms, …) can be revised in our previous published paper (Kazakova et al., 2018). First, it must be pointed out that during the 2013–2016 period, samples from MAT sampling point were not available because the channel was dry.

Samples from ROC, BER, and LDP showed in general the lower (or null) values for most of the analytes. However, water samples from ROC (in 2014) showed 5.1 ng mL^{-1} of CPR and this drug was also detected in both muscle and viscera *P. clarkii* samples from this location. Despite the high value detected in the water sample, the low level of CPR in the crayfish specimens (<1.0 ng g^{-1}) could be indicating a recent discharge. CPR is also a usual veterinary drug thus its presence could be justified by livestock farming activities.

FMQ was found all years, excepting 2011, in water samples and crayfish specimens collected from AJO sampling point; it is noticeable the high values found in the years 2014–2016 in the water samples with values of 4200 and 5100 ng mL^{-1}, respectively. Additionally, high levels of FQM have been measured for the captured specimens in this location, which could be indicative of a continuous discharge on sampling dates. For the rest of the years excepting 2015, low FMQ water levels were also accompanied by relatively high values from *P. clarkii* specimens. In 2015, the low FMQ level in water from AJO (<2 ng mL^{-}1) and the relatively high levels of this drug detected in the muscle and viscera of captured crayfishes could be correlated to a non-recent discharge, being its accumulation in the specimens the confirmation for their selection as bioindicators. In this location, the important equine and bovine livestock farming could justify this fact since flumequine is a widely used veterinary antibiotic (Biel-Maeso et al., 2018; Kim et al., 2018; Ma et al., 2012).

Additionally, other drugs (IBU and CPR) were found at lower concentration levels in water samples and/or specimens over the years in this location showing that AJO could be considered as the location that supports the greater environmental pressure from pharmaceuticals into the Doñana National Park. The relatively high persistence of IBU in the environment could be indicating the influence of the discharges from some of the WWTPs that are located in the DNP surroundings. It is noticeable that this drug was only detected in this location; its detection in water samples from LPD could be explained in other terms (see below in the text).

In the year 2014, the sampling was carried out after the annual peregrination to “el Rocio” that cross the DNP and gathers more than one million people in the village of “El Rocio” for some days. This fact could explain the detection of important levels of ciprofloxacin (CPR) in the waters from ROC (close to this village) due to it is an antibiotic widely used for the treatment of several infectious diseases and important amounts of no metabolized drug is excreted through the urine.

The punctual presence of carbamazepine in some sampling points and years must be directly related to the sporadic influence of effluents containing human excretions, probably from discharges of the WWTPs that are located around the Park. Carbamazepine appears recurrently in several studies related to the occurrence of PECs in urban wastewaters (Martínez-Piernas et al., 2018; Fekadu et al., 2019). CBZ was detected in specimens of *P. clarkii* from MAT (in 2012), BER (in 2015) and PAR (in 2015 and 2016). An important level of CBZ (4.7 ng mL^{-1}) was also measured in the water sample from BER in 2015 but this drug was undetected in the crayfish specimens. It is remarkable that this drug was detected at high levels in effluents from urban wastewater treatment plants (WWTPs) located in the surroundings of the DNP (Camacho-Muñoz et al., 2010a; Camacho-Muñoz et al., 2010b). The low environmental degradation of CBZ (Bahlmann et al., 2014) could explain its presence in crayfish specimen in deep areas of the Doñana National Park from the surrounding towns.

Finally, the detection of salicylic acid and ibuprofen in water samples from Lucio de Palacio (years 2012, 2015 and 2016) could be related to sporadic discharges from DNP scientific personnel and some of the park guards. Acetylsalicylic acid and ibuprofen are two very common pharmaceuticals usually detected at high levels in urban wastewaters, including those located near DNP (Camacho-Muñoz et al., 2010a; Camacho-Muñoz et al., 2010b). Both drugs, like other active pharmaceutical products, have a medium to high persistence in the environment, as a matter of fact advanced oxidation processes have arisen as alternatives for their degradation in wastewaters (Arthur et al., 2018; Jallouli et al., 2018; Ellepola et al., 2020).

4. Conclusions

A study on the presence and occurrence of 23 selected pharmaceuticals in crayfish and water samples from Doñana National Park (Spain) was carried out from 2011 to 2016 period. This study demonstrated that some pharmaceuticals were detected even at relatively high levels. It is remarkable that their occurrence in specimens of *P. clarkii* could indicate the persistence of the discharge sources.

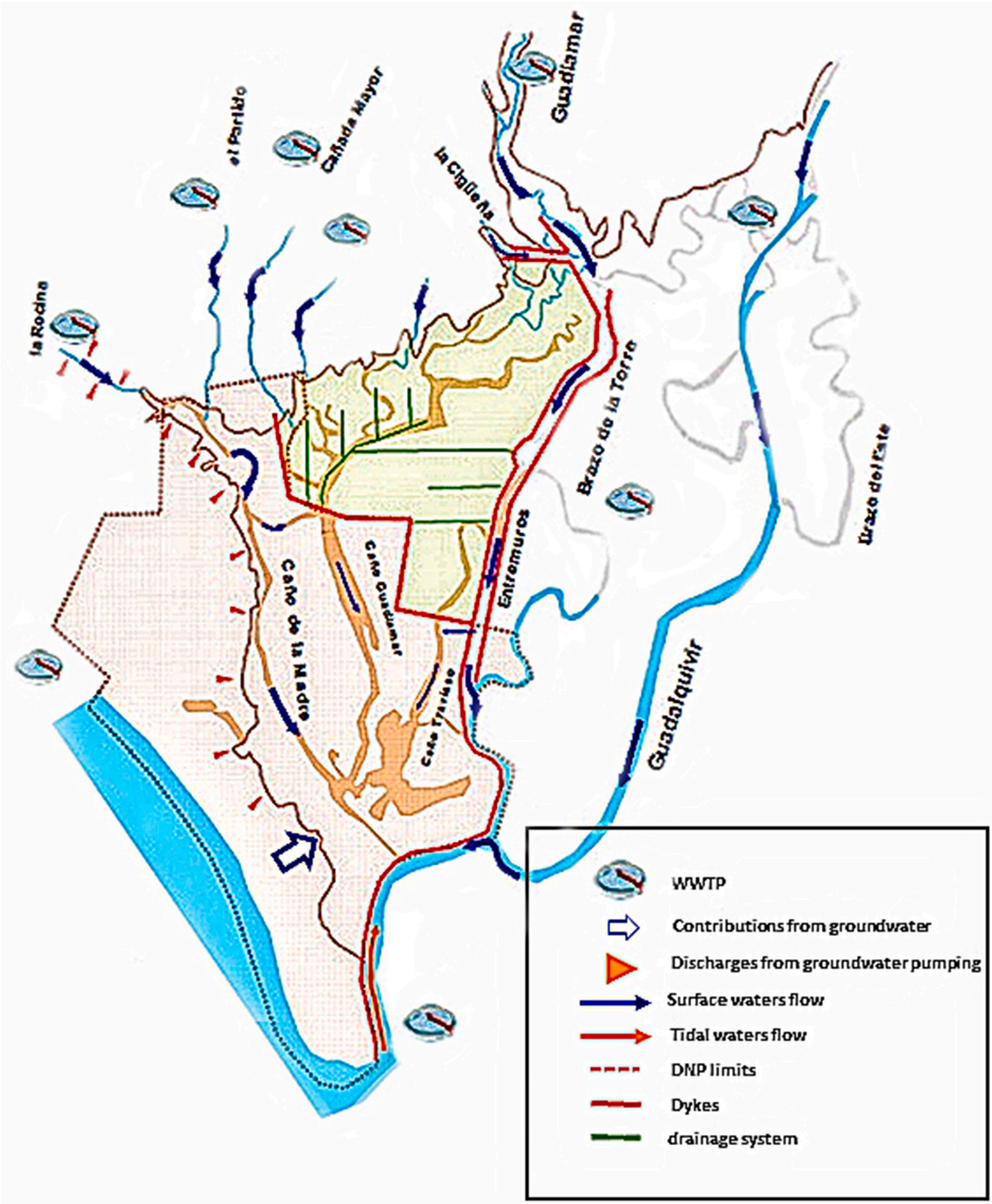

Fig. 2. Hydrological functioning diagram of the Doñana National Park and its surroundings and locations of urban wastewater treatment plants (WWTPs).

Table 1
Levels found (water (ng·mL^{-1})/muscle (ng·g^{-1})/viscera (ng·g^{-1})) for the selected analytes from the different sampling points.

Sampling point												
Analyte	Year 2011						Year 2012					
	ROC	**BER**	**PAR**	**AJO**	**MAT**	**LDP**	**ROC**	**BER**	**PAR**	**AJO**	**MAT**	**LDP**
IBU												**<3/0/0**
CBZ											**0/<0.9/<0.9**	
SMX											**0/<0.3/<0.3**	
FMQ			**<2/<1.1/<1.1**							**<2/<1.1/<1.1**		
SAC								**<0.8/0/0**			**<0.8/0/0**	**<0.8/0/0**
CPR			**<2/0/0**	**0/<1.0/<1.0**								
	Year 2013						**Year 2014**					
	ROC	**BER**	**PAR**	**AJO**	**MAT**	**LDP**	**ROC**	**BER**	**PAR**	**AJO**	**MAT**	**LDP**
IBU				**<3/0/0**	**					**<3/<1.5/0**	**	
CBZ					**						**	
SMX					**						**	
FMQ				**0/<1.1/<1.1**	**					**4.2/21.2/1.1**	**	
SAC					**						**	
CPR					**		**5.1/<1.0/<1.0**				**	
	Year 2015						**Year 2016**					
	ROC	**BER**	**PAR**	**AJO**	**MAT**	**LDP**	**ROC**	**BER**	**PAR**	**AJO**	**MAT**	**LDP**
IBU					**						**	**<3/0/0**
CBZ	**0/10.8/0**	**4.7/0/0**	**0/0/14.6**		**				**0/9.6/20.8**		**	
SMX					**						**	
FMQ				**<2/30.6/27.6**	**					**5.1/26.2/34.2**	**	
SAC					**	**<0.8/0/0**					**	
CPR					**						**	

0: Undetected in the corresponding sample.
** Water samples and P.clarkii specimens not available.
Blank: undetected in any sample.

The data analysis correlating the pharmaceutical found and the environmental issues of the corresponding sampling points reveals that the main source of pharmaceuticals into DNP might be the livestock farming activities, mainly equine and bovine. Additionally, results reveals that some sampling points might also be submitted to the influence of urban areas since the presence of some drugs commonly prescribed for human use have been also verified. However, despite the high levels found in WWTPs effluents by some authors (Camacho-Muñoz et al., 2010a; Camacho-Muñoz et al., 2010b) and that surface waters that contain discharges from urban WWTPs penetrate to deep areas of the Park, the influence of urban wastewaters from surrounding villages does not seem to be very important according to the data obtained in this study.

Finally, a direct anthropogenic influence was also confirmed by the data obtained during the annual pilgrimage to "el Rocio" in 2014 which passes through DNP and by the data from LDP sampling point where the close presence of DNP staff could exert some environmental pressure.

The data from captured *P. clarkii* specimens reveals that this organism could be a good bioindicator because although in some sampling points water samples analysis does not reveals the presence of any of the selected pharmaceuticals, crayfish specimens accumulated some of them into their muscle and/or viscera.

Credit author statement

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2021.113314.

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