Convocatoria de ayuda a proyectos de investigación liderados por jóvenes investigadores (9ª ed., 2019)

1. Datos de identificación.

Título de la propuesta	TEMLAT": Temperature sensitivities of biofilm enzymatic activities: a latitudinal experiment
Categoría	Ganando Independencia
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2. Memoria Técnica. Actividades y resultados de investigación

2.1. Introducción (Planteamiento, objetivos y justificación)

Global warming predictions suggest an increase of mean air temperatures because of human activities (IPCC 2021). Global surface temperature was 1.09 [0.95 to 1.20] °C higher in 2011–2020 than 1850–1900, with larger increases over land (1.59 [1.34 to 1.83] °C) than over the ocean (0.88 [0.68 to 1.01] °C) (IPCC, 2021). Particularly, the temperature increased are not expected to be equal in all the latitudes. It is reported that climate change is occurring faster in high-latitudes regions, specifically the Arctic is warming more than six-fold faster than the global average (Huang et al. 2017).

Despite occupying small geographical area, streams play a key role in global carbon cycle, thanks in part to river biofilms who play a key role in degradation of organic matter and nutrient cycling (Battin et al. 2016). The extracellular enzymes released by microbial biofilms, are a primary mechanism for organic matter decomposition since they transform polymeric molecules into low-molecular-weight molecules that can be assimilated for bacteria (Sinsabaugh, Hill, and Shah 2009). Most of the used extracellular enzymes in aquatic environments are those which degrade phenols, organic nitrogen and phosphors and polysaccharides, linked to C, N, and P recycling. Microbial activity is highly responsive to temperature increase, but this response could change depending on the temperature sensitivity of microbial communities adapted to different climates. Water river warming significantly affects biofilm metabolism, increasing primary production and

community respiration, as well as increasing microbial biomass, activity, and production (Freixa et al. 2017; Ylla, Canhoto, and Romaní 2014). However, previous experiments incubating samples at a wider range of temperatures showed a non-linear significant response of temperature on biofilm metabolism (Boulêtreau et al. 2012) and other studies in soils reported latitudinal changes, showing how greater temperature sensitivity in extracellular enzyme activities in soils from cooler climates than in warm-adapted zones (German et al. 2012).

For that, this project aimed to understand the temperature sensitivity of extracellular enzyme activity of aquatic biofilm in different latitudinal environments. This information until now remains unexplored for enzymes in aquatic ecosystems as well as it is unclear how microbial communities adapted to local regimes respond to temperature changes in terms of organic matter degradation. We studied five sampling sites across a latitudinal gradient in the north hemisphere (from 5ºN to 69ºN) using a coordinated methodology. We measured extracellular enzyme activity linked to C, N and P organic matter sources acquisition incubated at five different temperatures ranged from 4ºC to 32ºC.

2.2. Descripción de la ejecución- Metodología

River biofilms and water were collected in streams not affected by morphological alterations neither pollution. The samplings were conducted between summer to autumn 2020. We select 5 different streams following a latitudinal gradient that included five different biomes, 2 sites in Canada (69ºN and 53ºN; Arctic and Boreal), Denmark (56ºN; Temperate), Spain (41ºN Mediterranean) and Sri Lanka (5ºN Tropical) (Figure 1).

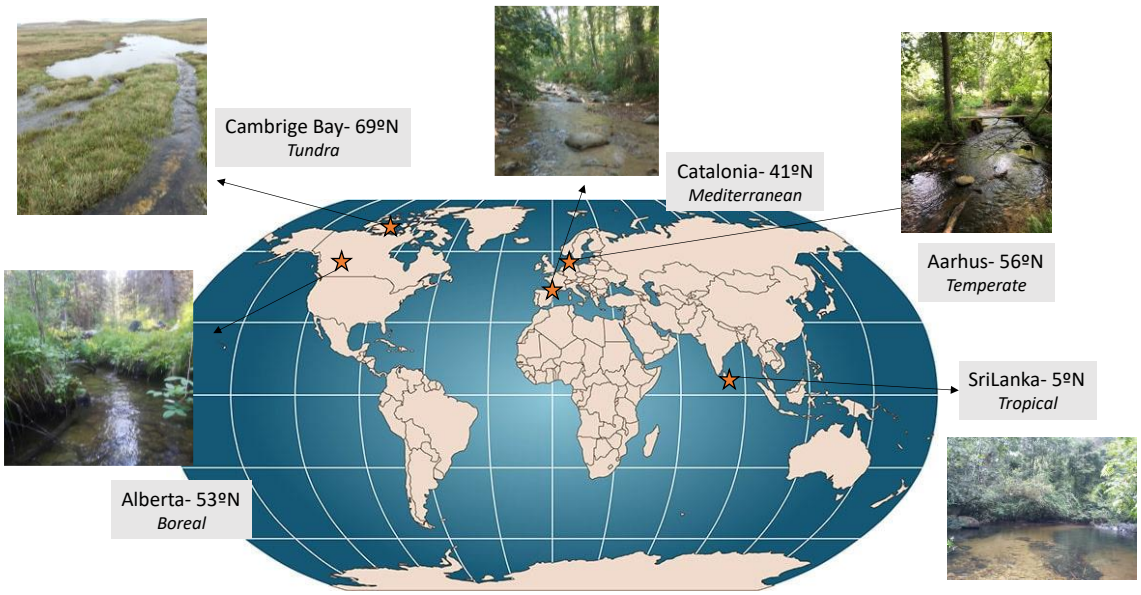

Figure 1. Map of the different sampling sites and different climate associated.

In each sampling site we collected and filtered triplicate water samples for the analysis of nutrients (NH4+, NO3-, SRP, DOC and TDN and DIN) cations and anions (Fe,S,Mg, Na, Si, K, Ca, P). Stream discharge was estimated using a mass balance approach by recording downstream changes in conductivity over time after a NaCl slug addition. Physicochemical parameters (temperature, dissolved oxygen, conductivity, and pH) were measured at each sampling site, using a multiparameter probe. For each stream, we also collected four stones for the biofilm characterization. In the laboratory the biofilm was brushed with stream filtered water and we estimated the surface area scraped. For each biofilm extract we measured the ash-free dry mass (AFDM), chlorophyll-a pigments, bacterial density, and the extracellular enzyme activities.

Extracellular enzyme activities incubations.

We measured the activity of 6 different enzyme activities using the biofilm extracts for each sampling site for each collected stone (Table 1). Samples were incubated at 5 different temperatures 4ºC, 8ºC, 16ºC, 24ºC, and 32ºC and at 5 different substrate concentration, 0, 0.05, 0.1, 0.3 and 0.8 mM during 1h to 4h depending on the enzyme and temperature incubation. EEA were incubated following the method described in Pastor et al. 2019. The total number of processed samples were 5 sites x 6 enzymes x 5 temperatures x 5 substrates concentrations x 4 replicates = 3000 samples. Enzyme activities were determined spectrofluorometrically using artificial fluorescence linked substrates (MUF and AMC), fluorescence was measured using a

fluorimeter microplate reader and activities expressed as the rate of MUF or AMC released for hour in relation to biofilm surface area. In the case of phenol oxidase activity absorbance was measured at 460nm using a spectrophotometer. And values were expressed as µmol DIQC cm^2 x h.

Table 1. List of enzymes, metabolism involved and concentrations used.

Enzyme	Artificial substrate	Involved in the metabolism of	Concentrations
β-D-glucosidase	MUF-β-D-glucopyranoside	C; last step of cellulose decomposition	0.05-0.8 mM
β-D-xylosidase	MUF-β-D-xylopyranoside	C; last step of hemicellulose decomposition	0.05-0.8 mM
Cellobiohydrolase	MUF-cellobioside	C; degradation of cellulose	0.05-0.8 mM
Leucine-aminopeptidase	Leu-AMC (L-leucine-4-methyl-7-coumarinylamide)	N, C; decomposition of peptides	0.05-0.8 mM
Phenol oxidase	L-DOPA	C; Degradation of lignin	0.6 -3 mM
Phosphatase	MUF-phosphate	P; decomposition phosphomonoesters	0.05-0.8 mM

We also calculated different enzyme ratios ((GLU+XYL+CEL):PHOS, (GLU+XYL+CEL):LEU)) which are used as an indicator of the greater or lower capacity of microbial community to degrade carbon organic matter in contrast to its capacity to degrade phosphorus or nitrogen organic matter.

Then, we measured the Michaelis-Menten kinetics (maximal rate of velocity, *Vmax*) and half-saturation constant (*Km*) for each enzyme at each of measured temperatures using the following non-linear least square regression:

$$EA = \frac{Vmax \times S}{Km + S}$$

Where *EA* is the enzyme activity, *S* is the substrate concentrations, *Km* is the substrate concentration at half-maximal velocity and *Vmax* is the maximal velocity. Regressions were performed using the "nls" function in R studio, R software version 4.0. Estimate parameters *Vmax*, *Km* and *p-value* were extracted from each regression. Then *Vmax* were log-transformed and linear regressions were plotted and fitted as a function of temperature for each site and

activity. Slopes and intercepts were calculated fitting a linear model, "lm" function in R software. Slopes of *Vmax* linear regressions, representing the temperature sensitivities of each enzyme kinetic parameter, were also expressed as Q_{10} values according to Q_{10} = exp (slope x 10).

2.3. Resultados obtenidos (cumplimiento de objetivos)

Objective 1. Determine the potential EEA (at substrate saturation concentrations and non-limiting temperature) in river biofilms across a latitudinal gradient. Completed 100%

Sampling sites characterization

Environmental variables were clearly different among sampling sites as is shown in Figure 2 and Table 2. Higher values of water temperature were observed at the tropical site in Srilanka (6º N) with values around 26ºC. Higher latitude sites (Artic-69ºN and Alberta-56ºN) were characterised by high values of DOC (ranged between 6.8 to 9.8 mg/L) and Mediterranean (41ºN) and Aarhus (56ºN) sites were characterised by high nitrogen content (nitrate concentrations ranging from 1.3 to 2.4 mg/L) (Fig.2, Table 2).

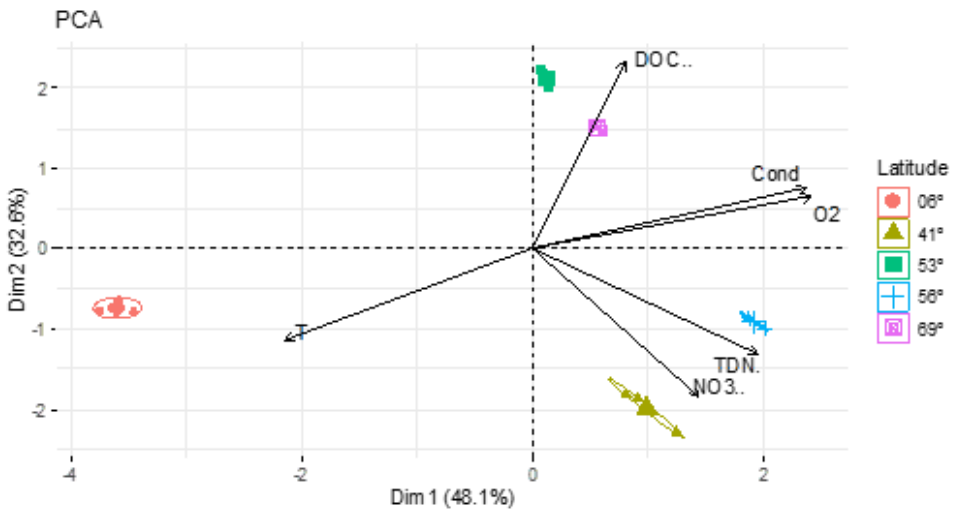

Figure 2. Principal components Analysis (PCA) based on environmental data from each of the 5 sampling sites ordered by latitude. Only significant correlated variables were plotted. T= Temperature, NO3= Nitrate, TDN= Total dissolved nitrogen, DOC= Dissolved organic carbon, Cond= Conductivity, O2= Oxygen.

Table 2. Physicochemical parameters in water (data are mean values) for each sampling site.

		NH4	PO4	NO3	DOC	TDN	DIN	FV	Cond	T	O2
Site	**Latitude**	*mg/L*	*mg/L*	*mg/L*	*mg/L*	*mg/L*	*mg/L*	*m/s*	*µS/cm*	*ºC*	*mg/L*
Cambrige Bay	69ºN	0.029	0.004	0.001	6.848	0.476	0.029	0.518	513.333	12.200	10.404
Alberta	53ºN	0.025	0.010	0.006	9.894	0.372	0.031	0.202	455.667	10.700	8.001
Catalonia	41ºN	0.077	0.088	1.273	1.447	1.497	1.351	0.598	377.750	16.100	7.580
Srilanka	5ºN	0.039	0.007	0.143	0.857	0.121	0.182	0.243	27.000	24.300	1.567
Aarhus	56ºN	0.033	0.023	2.423	1.807	3.163	2.457	0.285	451.667	9.867	10.757

NH4=Ammonium, PO4=Phosphate, NO3= Nitrate, DOC= Dissolved organic carbon, TDN= Total dissolved nitrogen, DIN= Dissolved inorganic nitrogen, FV= Flow velocity, Cond= Conductivity, T= Temperature, O2= Oxygen.

Potential enzymatic activity across a latitudinal gradient

We selected enzymatic activities at 16ºC (as non-limiting temperature) and saturated concentrations of each substrate (0.3mM for X, G, L and P enzymatic activities, 0.8 mM for C and 1.5mM for PX) to observed differences in the potential enzymatic activity across latitudes. Our results showed how enzyme activity related with organic carbon degradation (i.e G: β-glucosidase activity and C: Cellobiohydrolase activity) showed a clear decreasing pattern as latitude increased except for the Artic site where microbial activity was quite higher. Remarkably higher concentrations of leu-aminopeptidase and phosphatase activities (enzymatic activities related with N and P organic carbon degradation, respectively) were also observed in the Artic site (69ºN) in contrast to the sites located in lower latitudes (Figure 3). Enzymatic activities recorded in the Artic site were relatively higher than those found in previous studies for similar latitudes at high Artic streams (Pastor et al. 2019, 2021).

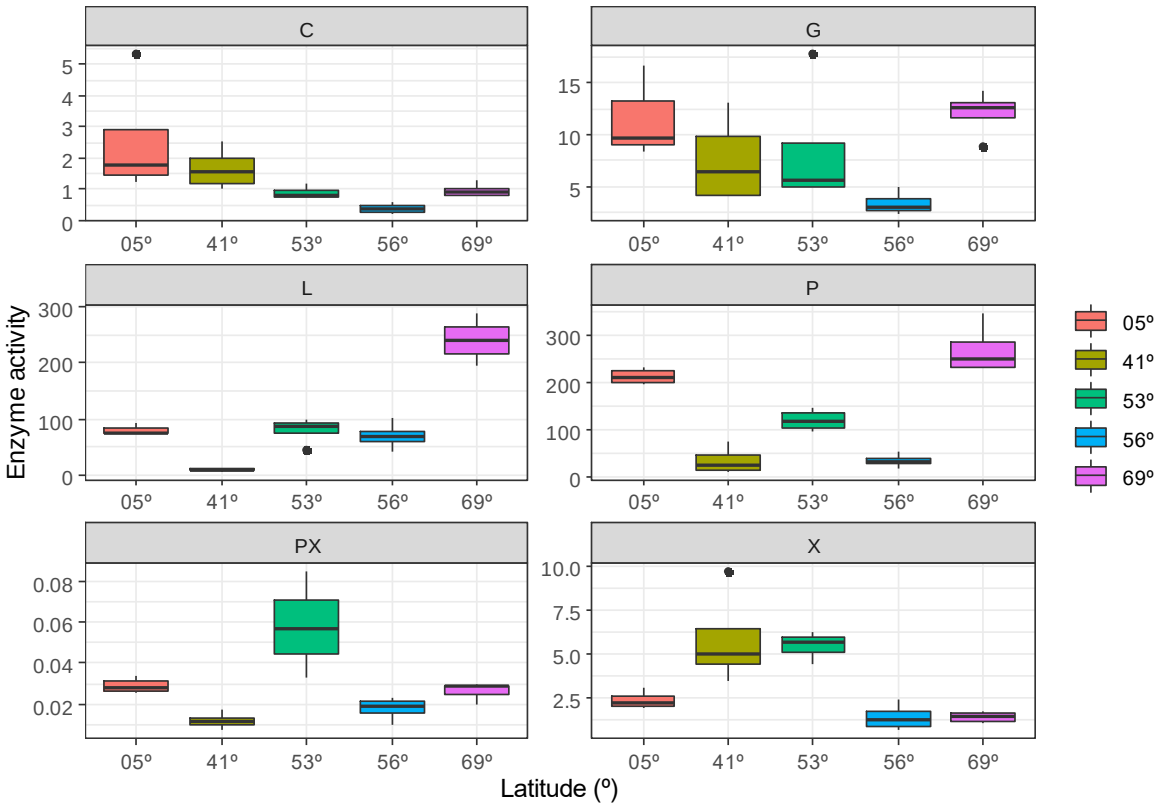

Figure 3. Results of enzymatic activities (nmol MUF or AMC/cm^2 x h) for each substrate at 16ºC at saturated concentrations for each sampling site order by latitude. C= Cellobiohydrolase activity, G= β-glucosidase activity, L= Leucine-aminopeptidase activity, P= Phosphatase activity, PX= Phenol oxidase activity, X=Xylosidase activity.

Objective 2 and 3. Assess the enzyme temperature sensitivity in river biofilms including their kinetics parameters depending on the geographic zone and depending on the specific enzyme considered. ***100% Completed***

The *Km* and *Vmax* were calculated for each enzyme, site, and temperature resulting on 150 *Km* and *Vmax* values after the non-linear regressions fitted to a Michaelis Menten curves (Figure 4, as an example). Results clearly showed a hyperbole increased of activity as temperature and concentrations increased.

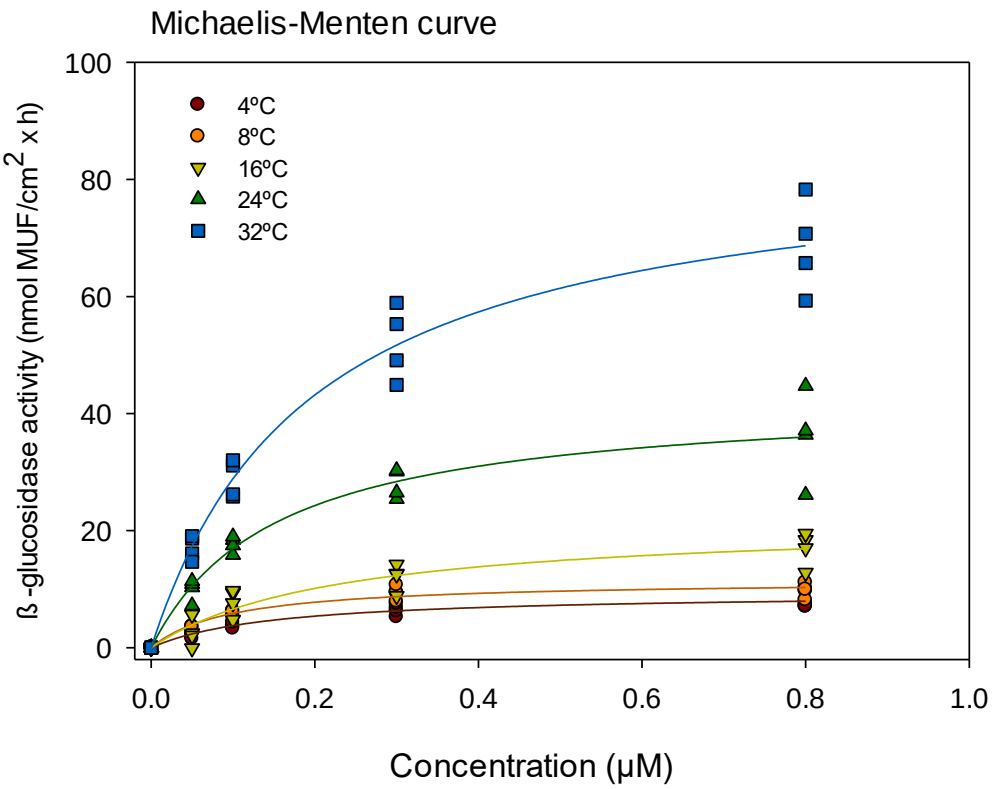

Figure 4. Michaelis Menten curve for β-glucosidase activity of samples for Mediterranean river (41º) for each of the incubated temperature.

Then, we examined the temperature sensitivity of *Vmax* in the different regions (Figure 5). Overall *Vmax* linear increased with temperature for all enzymes at all sites (with significant p-values) except for few cases that did not follow linear model (Figure 5). The magnitude of temperature response of *Vmax* across enzymes and sites ranged from 0.0036 to 0.048 $^{o}C^{-1}$ corresponding to the slope of the linear model. The extracellular enzymes measured showed a distinct sensitivity to temperature depending on each region and enzyme. Specifically, rates of decomposition of organic carbon (C and G activities) were more sensitive to temperature at high latitudes as slopes were higher than at lower latitudes which also higher Q_{10} values (1.42 vs 1.34 respectively for G). N and P organic decomposition showed different patterns which high slopes values (and Q_{10}) at 5ºN latitude (Sri Lanka site) than other sampling sites for both L and P activities (Figure 6). These results indicated that the degradation of organic nitrogen and phosphorus compounds were faster in lower latitudes in contrast to the degradation of organic carbon which was faster at higher latitudes sites (Figure 6) indicating a different nutrient limitation depending on the latitude. In the case of phenol oxidase activity, Q_{10} values ranged from to 0.84 to 1.23 which high vales at Artic site (69ºN) showing lower values of Q_{10} than the other enzymes. These results are not in

accordance with previous studies that observed how phenol oxidase activity tends to have higher temperature sensitivity than hydrolytic enzymes (Pastor et al. 2021; Wang et al. 2012).

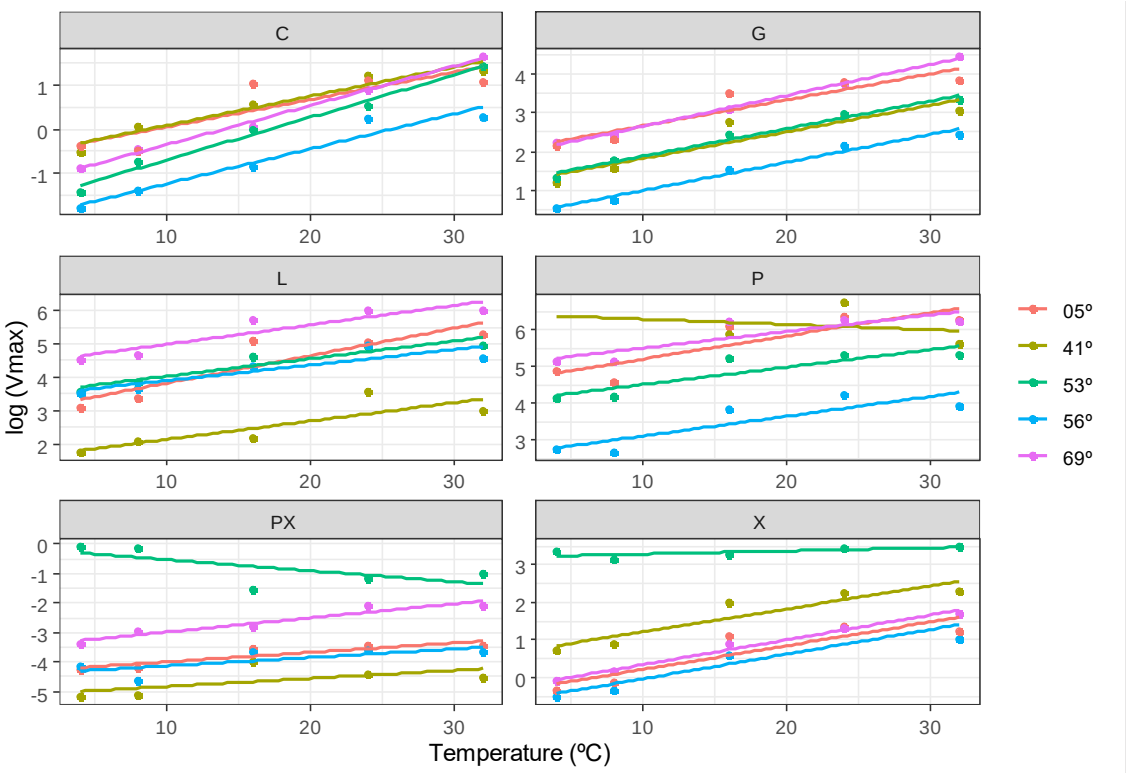

Figure 5. Enzyme activities Vmax plotted as a function of incubated temperature in each of the different sampling sites. C= Cellobiohydrolase activity, G= β-glucosidase activity, L= Leucine-aminopeptidase activity, P= Phosphatase activity, PX= Phenol oxidase activity, X=Xylosidase activity

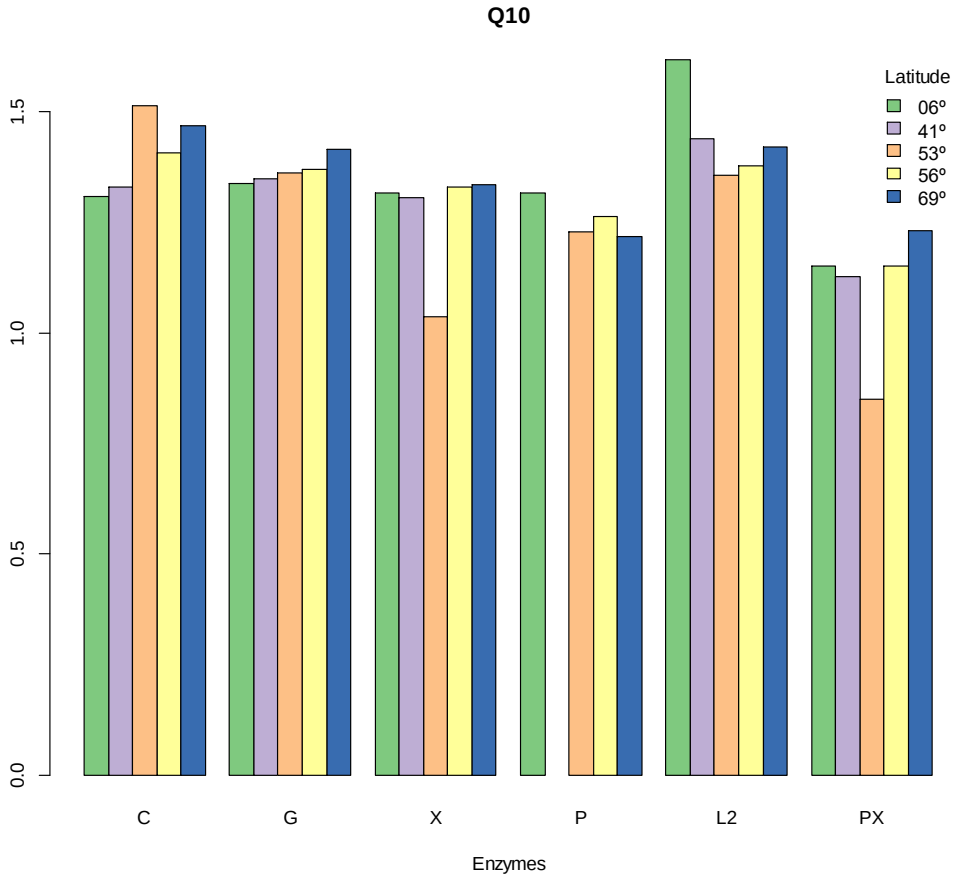

Figure 6. Q_{10} results for each enzyme activity and sampling site (latitude). C= Cellobiohydrolase activity, G= β-glucosidase activity, L2= Leucine-aminopeptidase activity, P= Phosphatase activity, PX= Phenol oxidase activity, X=Xylosidase activity.

2.4. Conclusiones y valoración de la ejecución

Our results showed a remarkably higher temperature sensitivity in the Artic region (69ºN) than sampling sites located at lower latitudes, especially showing higher leu-aminopeptidase activity (organic nitrogen compounds degradation or degradation of peptides) and phosphatase activity (organic phosphorous compounds degradation/ or degradation of organic phosphorus compounds) at that site, probably indicating limitation of that nutrients (N and P) at higher latitudes in contrast to carbon. Moreover, we observed that activity of enzymes related with organic carbon degradation (β-glucosidase and Cellobiohydrolase activity) clearly decreased as latitude increased, indicating a C-limitation at lower latitudes. The results of TEMPLAT project also indicated that the degradation of organic N and P compounds were more sensitive to

temperature in lower latitudes in contrast to the degradation of organic carbon which was higher sensitivity at higher latitudes sites. Our results help to improve the prediction about temperature responses of organic matter degradation involving C, N, P degradation to global warming in river systems around the world, showing different responses in nutrient stoichiometry depending on the latitude of the river sampling sites.

Final remarks-

The half of the planned sampling sites (6/12) proposed in the initial project had cancelled due to COVID-19 restrictions (Florida, Colombia, Kenya, Argentina, and Antarctica). After some postponements and due to different logistical problems, these sampling sites had finally cancelled and did not include it in the project. Thus, for the same reason, the sampling chronogram and some of the initial expenses had been modified from the initial proposal.

2.5. Publicaciones resultantes

At present, there are 2 scientific publications in preparation related to the TEMPLAT project, the tentative titles are:

- Freixa et al. *Temperature sensitivity of river biofilms enzyme activities across a latitudinal gradient.*
- Pastor et al. *The Michaelis–Menten kinetics of aquatic extracellular enzymes in response to temperature.*

Moreover, results of the project have been presented at an international conference: **ASLO virtual meeting, 22-27 June 2021**, with an **oral presentation** entitled: "*Temperature sensitivity of river biofilms enzyme activities across a latitudinal gradient"* authored by: Anna Freixa, Núria Catalan, Pau Gimenez Grau, Dani D. Quijano, Tenna Riis, Anna M. Romaní, Suzanne Tank, Lishani Wijewardene and Ada Pastor.

Fdo: ANNA FREIXA CASALS

en Girona, a 24 de Enero de 2022

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