# Assessment of ecological function indicators related to nitrate under multiple human stressors in a large watershed

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## ABSTRACT (343 WORDS):

Important ecological functions indicators, such as nitrate net balance (NNB), including nitrate removal (NR) and nitrate production (NP), seem to be impacted by human stressors and climate change, particularly during low flow periods associated with high nitrate pollution. NR is induced by in-stream processes such as nitrate uptake, denitrification, and transient storage. These processes are usually investigated by means of in-situ measurements, but the evaluation on a large scale is not yet developed. Hence, the objectives of this study are (i) to validate a model that quantifies NR indicator in a reach at a monthly time step in the Garonne watershed; (ii) use this model to quantify NR in all reaches at watershed scale; (iii) to analyze NR spatially and temporally at the reach scale, and finally (iv) to identify drivers influencing NR patterns. We used the Soil and Water Assessment Tool (SWAT) model to simulate the impact of human stressors such as land management and municipalities effluents on in-stream nitrogen cycles. The results show the seasonal variation of NR and NP ranging between –1.77 $gN.m^{-2}.d^{-1}$ and +1.62 $gN.m^{-2}.d^{-1}$. NR is stronger during the spring and summer periods (median of -0.1 $gN.m^{-2}.d^{-1}$). The hot spots of NP are located downstream in the main rivers whereas NR strongly occurs in small reaches of lowlands and intermediate streams defined by a Strahler order between 3 and 5 and a slope under 0.5%. NR is stronger in hillsides areas such as forests, wetlands and surprisingly agricultural areas and NP increases down in the municipalities due to the effluents. The spatio-temporal variability of NR makes the validation of the model for reaches with Strahler number more difficult than the measured one. However, this study shows that the NNB dynamics in time and space depend on a combination of influencing factors (slope, discharge and hydraulic condition explained by Froude number and nitrate concentration). Investigating a relationship between NR, river characteristics and land management is a promising way to support stakeholders in water management decisions and increase awareness and involvement of people for sustainable management of water resources.

***KEYWORDS:*** water quality, ecological function, nitrate removal, nitrate production, modeling

## 1. INTRODUCTION

The most valuable ecosystem service in Europe is the regulation of freshwater and coastal water quality with a median value of 1,754 euros per hectare and year (Martín-López et al., 2018). This natural contribution is assessed by quantifying ecological functions such as denitrification (removing nitrate), suspended matter depletion (linked to erosion rate) or nutrient plant uptake (removal of nutrients). Some of these ecological functions may be relevant indicators of this service as suggested by several studies (Sauvage et al., 2018; Volk, 2015). Two of these related functions indicators are nitrate production (NP) and nitrate removal (NR). NP is the total quantity of nitrate produced in the contributing area of the reach whereas NR is the total quantity of nitrate withdrawn out of a reach. NR undergoes multiple pathways in natural ecosystems (Zarnetske et al., 2011). In the process of nitrogen cycling, nitrate can be carried out and diluted by water flows (runoff, free and hyporheic waters) or transformed by different biogeochemical processes such as plant uptake, denitrification, mineralization, nitrification or nitrogen fixation. The complexity of the nitrogen cycle, including physical, chemical and biochemical processes, is well understood in reaches (Gruber and Galloway, 2008), but is hardly measurable and rarely quantified in all reaches of an entire watershed. The quantity of nitrate in water depends not only on the physical and biological characteristics of land such as soil properties and topography, but also on the source or type such as groundwater, riparian zones, floodplains, lakes, and estuaries. Stressors such as agriculture, industries, cities, and dams will act as sources for nitrate whereas some ecological functions as denitrification will act as sinks for nitrate (Haag and Kaupenjohann, 2001). As defined in Sabater et al. (2019), the term stressor focused exclusively on the anthropogenic disturbances.

Studies that worked on NR were either field-based (Sauvage et al., 2018), involved integrated complex modelling at the plot scale (Jégo et al., 2008) or focused on a static study modeling at the watershed outlet (Grizzetti et al., 2015; Sutton et al., 2011). Such modeling approaches can lead to a high uncertainty rate related to the temporal variability in model simulations and forecasts (Birgand et al., 2009). The temporal and spatial variation of NR in water bodies is still not well investigated. There is a clear lack of integrative approaches to quantify water quality regulation services and related indicators in time and space at the watershed scale.

Nitrogen cycling in the aquatic system is well known but the quantification of NR through the different pathways remains partially developed (Grizzetti et al., 2015). In the river system at the scale of the reach (integrating the free water and the benthic part), nitrate can be produced or removed by several processes (García-ruiz et al., 1998). Considering the difference between input and output of a reach, the in-stream nitrate evolution at the scale of the reach is typically called the "Nitrate net balance" (NNB). Understanding this NNB variability in reaches through a watershed could help to determine areas with high potential of NR or NP. Therefore, the determination of "hot spots" and "hot moments" of NR may be improved by a better assessment of this variability. This terminology was developed by McClain et al. (2003) in order to highlight special areas and periods of time where functions such as denitrification or NR are more intensive. Global sampling strategies of nitrate concentrations are difficult to achieve on a watershed scale because of high costs and logistic issues. To this end, modeling approaches can solve these difficulties and used to quantify nitrate trends in time and space. Numerous dynamic nitrogen models exist and can be differentiated by the scale of implementation (stream, watershed, region) (Sutton et al., 2011). However, NNB quantification requires a mechanistic model (1) simulating daily water balances and in-stream processes at large scale and (2) including human impacts such as

agriculture, dams and municipalities management. Annual step models and watershed mass balanced models as Global-NEWS, Green, MONERIS, Polflow, and Sparrow (Sutton et al., 2011) cannot be used to predict seasonal variations or upstream-downstream gradients of the ecological functions. Among the models being able to simulate at the daily time step, some are functioning on grid cell resolution (INCA) and others have never been tested at large scale (RiverStrahler; (TRK)/HBV-NP) (Sutton et al., 2011). From the investigated models, only the Soil and Water Assessment Tool (SWAT; Neitsch et al., 2011) fulfills the spatial and temporal criteria and integrates the effects of stressors such as dams, incised streams in an agricultural area, channelized river, urban stream. The model outputs analysis should enable the quantification of in-stream NR indicator and its behavior in time and space in order to identify "hot spots" and "hot moments".

Modeling these processes is necessary not only to understand NR functioning in reaches along the watershed but also to identify influencing factors on a large scale. Control factors as geomorphology, weather, type of soil, contaminant concentration can be used indirectly to explain NR. Therefore, defining these influencing factors could help to improve the understanding of the related processes and their feedbacks in the context of climate change.

In this study, we focus on the spatial and temporal dynamics of the water quality regulation service by modeling some indicators such as NR in the freshwater ecosystems (free water and benthic and hyporheic zone) of the Garonne river. The objectives of this study are (i) to validate a model that quantifies the indicator of NNB in a reach at a monthly time step allowing the identification of different sources and sinks of nitrogen as ecological functions of this system, (ii) use this model to quantify NNB in all reaches of the Garonne River; (iii) to analyze space and time dynamics of the NNB in rivers at the reach scale and monthly time step, and (iv) to identify drivers controlling the dynamics of the NR process.

101 The paper is organized as follows. Section 2 describes the procedure used to quantify
102 and analyzed the NNB indicator in Garonne watershed and to determine the potential
103 influencing factors that might explain NR. Sections 3.1 and 3.2 present the results of the
104 modeling performance for predicting NNB and its dynamics. Section 3.3 focuses on the
105 influencing factors that explain NR. Section 4 finally discusses the results in a general context
106 and with regard to water management application.

107 ## 2. MATERIALS AND METHODS

108 ### 2.1. Study Area

109 The Garonne watershed, located in Southwestern Europe, is a well-investigated area with
110 available information on climate, geomorphology, soils, slope gradients, land uses, and
111 multiple human-driven stressors (Espitalier-Noël et al., 2016; Grusson et al., 2018). The
112 Garonne River is France's third longest river and has its source in the Spanish Pyrenean
113 massif and discharges into the Atlantic Ocean (near Bordeaux in France). The Garonne is
114 eutrophic in the middle and lower watershed where the most active biological activities occur
115 on the natural river bed as epilithic biofilm (Ameziane et al., 1999).

116 It is divided into three geographic entities: the *plain* surrounded by the *Pyrenees* in the
117 South and the *Massif Central* in the North-East (Tockner et al., 2009). The watershed is
118 located between different climatic zones, with Mediterranean climate conditions in the East of
119 the watershed, a continental type in the South, and with an oceanic climate in the North and
120 West. In the decade between 2000 and 2010, mean river discharges of 600 $m^3. s^{-1}$ were
121 recorded at the outlet at Tonneins (the last gauging station not influenced by the tide,
122 corresponding to a drainage area of 50 000 km²). The daily flow variations are strongly
123 influenced by 210 dams that have a capacity of 3.2 billion cubic meters of water (Sauvage et
124 al., 2003). Agriculture (60 %) dominates the lowland plain whereas the upland is mainly
125 covered by forest (32%) and pasture. Settlement and infrastructure areas represent 2.5% of the

126 total surface. The watershed's population is about five million causing 3639 tons per of
127 nitrogen municipalities effluents per year.

128 ## 2.2. Model description

129 SWAT was developed to quantify the impact of land management practices in large,
130 complex watersheds with varying soils, land use, and management conditions over a long
131 period of time (Arnold and Fohrer, 2005). It is a conceptual model that operates on a daily
132 time step.

133 The inland and instream nitrogen movement and transformation are simulated as a
134 function of the nitrogen cycle (Jha et al., 2004; Ullrich and Volk, 2009). Nitrogen is added to
135 the soil by fertilizer, manure or residue application, fixation by bacteria, and rain (Neitsch et
136 al., 2011). Nitrogen losses occur by plant uptake, surface runoff in the solution and the eroded
137 sediment (Jha et al., 2004; Ullrich and Volk, 2009). The dynamics of nitrate movement in the
138 river bed of each reach are based on the QUAL2E model (Brown and Barnwell, 1987) which
139 integrates the volatilization, addition, mineralization, nitrification and denitrification
140 processes (Neitsch et al., 2011). The QUAL2E module, integrated into the SWAT model,
141 considers all significant factors including the major interaction of the nutrient cycles, algae
142 production, benthic oxygen demand, carbonaceous oxygen uptake, atmospheric aeration and
143 their effects on the behavior of dissolved oxygen. The model depends on nitrogen dissolution
144 in reaches and their adsorption in sediments. Dissolved elements are carried by flows whereas
145 adsorbed ones are dropped off the bottom of the river. In-Stream processes are divided into
146 two parts: biologic and physical processes. The biological component is driven by the
147 QUAL2E module whereas the physical component is linked to river connectivity with ground,
148 sub-surface and surface waters, through the hyporheic zone as an in-stream process simulated
149 by SWAT. These hydrological connectivities depend on the morphology of the river
150 (Stewardson et al., 2016) and gradient of river/groundwater water levels (Brunner et al.,

2017). The biological component includes two compartments that cannot be dissociated: the water column, an in-stream nitrogen cycle that is incorporated by the QUAL2E module (Brown and Barnwell, 1987) and the benthic part, modeled by the benthic module, which it is integrated into QUAL2E module. In the model, it is possible to deactivate the in-stream biological processes that only permits physical processes to occur. To simulate the movement of water bodies at the reach scale in time, the model stores a percentage of water and nutrients within a certain time span and restore according to the residence time of the reach. During a time step, there is no interaction between various water compartments of groundwater, lands, and reaches. The difference between loads integrates only the geochemical and physical processes that happen in the river. More details of the SWAT nitrate module can be found in Brown and Barnwell (1987) and Neitsch et al. (2011).

### 2.3. Model inputs

SWAT uses several inputs such as climate, topography, land use and soil data (see Table 1). Moreover, Grusson et al. (2018) showed that using climate dataset SAFRAN (Table 1) in SWAT modeling improves hydrological performance.

The annual outfall volume of a wastewater treatment plant and the amount of pollution in nitrogen produced by the agglomeration were calculated by using the European Database (UWWTP) and the method (based on a regression) suggested by Zessner and Lindter (2005) (more details can be found in Supplemental Material A). According to previous studies (Tisseuil et al., 2008; Tockner et al., 2009), in the case of the Garonne watershed, smaller agglomerations (less than 50 000 inhabitants) don't have a significate impact on the nitrate fluxes in the river. Hence, agglomerations with more than 50,000 inhabitants were selected as point sources (see Figure 1).

We also integrated eleven dams of the watershed with a reservoir volume ranging between 0.3 $km^2$ to 10.2 $km^2$ into the model. The data was provided by Electricité De France (project REGARD-RTRA/STAE).

Irrigation impacts hydrology and the leaching of nitrate into the river. Hence, based on the national statistics (SAA, 2017), we assumed that the following crops are irrigated: rice, olive, vineyard, fruit trees and berry plantations, maize, and almonds (Supplemental Material B).

### 2.4. Model setup

Based on land use and soil databases and watershed topography, 22 land classes, 13 soil classes, and the following 5 slope classes were defined: 0 -2, 2-5, 5-15, 15-25 and > 25% (Figure 2). The slope between 0-2% characterizes the riverine area. The surface runoff appears above a slope of 5% (Roose, 1996). The terrain with slopes between 2% and 15% is commonly cultivated and represents 50% of the area. The other 50% of the land is divided into equal grades; sloping (15-25%) and steep (>25%). With these classes, the SWAT model identified 1,320 subbasins with 49,460 HRUs. Such HRUs with land use, soil and slope classes that cover less than 10% of each subbasin were dismissed and assigned to other HRUs present in that particular subbasin, as suggested by previous studies (Grusson et al., 2018), giving a final number of 12,834 HRUs.

We compared the simulated NNB with two sets of observed data gathered from two independent Garonne watershed studies (Table 1). Both databases contain hourly values of $mgN.m^{-2}.h^{-1}$ (Table 1). It was necessary to convert it to daily values (in $gN.m^{-2}.d^{-1}$) to enable a comparison with the daily SWAT simulation outputs. We assumed that the NNB is homogenous over the day and multiplied the experimental value by 24 at t-time.

The first dataset with the related sampling protocol resulted from the study of Teissier et al. (2008). The samplings were taken from July 27 to 29 July 1999, at three sites of the

199 Garonne River downstream of Toulouse (seventh-order stream) (Figure 1). The Garonne
200 River is characterized by a "hyporheic zone" where processes occur only in the benthic part
201 (Teissier et al., 2008). Discharge and nitrate concentrations vary between 50 and 100 $m^3. s^{-1}$
202 and 5.9 and 21.3 mg. $L^{-1}$.

203 The second database was collected during the STREAMES European project (Martí et
204 al., 2004; Sánchez-Pérez et al., 2009) from 2001 to 2003 on every season. Twenty-four
205 experimental measurements of NR (in $mgN.m^{-2}.min^{-1}$) were carried out in two reaches of the
206 Lèze river (third-order stream) (Figure 1). These sites are characterized by low discharges
207 between 0.08 and 0.3 $m^3. s^{-1}$ and small nitrate concentrations (0.24 - 2.3 mg. $L^{-1}$). In this area,
208 previous studies showed that the processes of NR mainly occurred in the benthic part of the
209 river (Sauvage et al., 2018).

210 ### 2.5. Model calibration, validation and uncertainty analysis

211 For each step (validation and calibration), three years of initialization are carried out,
212 followed by a manual calibration of dam management, crop yield, and irrigation volume on an
213 annual scale. Hydrology and nitrate loads were automatically calibrated on a daily scale based
214 on previous uncertainty analysis. Model performance was evaluated by using standard
215 statistical criteria suggested by Moriasi et al. (2015, 2007), i.e. coefficient of determination
216 ($R^2$), Nash & Sutcliff Efficiency (NSE), and percent bias (PBIAS).

217 Unfortunately, the lack of data hampered a sensitivity analysis of dams and agriculture
218 management. That is the reason why a manual calibration was used to calibrate dam
219 management, crop yield, and irrigation volume on an annual scale. Parameters of this manual
220 calibration are summarized in Supplemental Material C. Auto-irrigation was applied in the
221 SWAT model. A satisfactory PBIAS value of -17 % was obtained between the simulated
222 volume of irrigation water and the recorded values in the Neste system (15.1 $Hm^3$ per year)
223 (Figure 1). Simulated yields of selected crops (Supplemental Material C) were similar to the

amounts provided in national statistics giving $R^2$ of 0.78 and a PBIAS of – 7% (Table 2) (SAA, 2017).

Model calibration on streamflow and nitrates loads was carried out on a daily scale at twenty streamflow gauges and fifteen nitrate monitoring stations for the period between 2000 to 2010 o (Figure 1). This included using the LH-OAT sensitivity analysis tool (van Griensven et al., 2006) and auto-calibration using the Sequential Uncertainty Fitting (SUFI-2) routine (Abbaspour et al., 2018) for streamflow and nitrate loads. The most sensitive parameters are provided in Supplementary Material C. Parameters are more sensitive for larger absolute t-stat values (significant if the P-values are close to zero). During the calibration period, we got a satisfactory quality of the daily simulation with an average of 0.54 for Nash-Sutcliffe criteria, 0.62 for regression coefficient, and -6.89 for PBIAS for the streamflow and 0.41, 0.68 and 9.69 % for the nitrate load. Table 3 and Table 4show the performance at the gauging stations respectively for streamflow and nitrate (Figure 1).

The model was validated for the period from 1990 to1999 on a daily scale to verify the model accuracy. The average NSE, $R^2$, and PBIAS of daily simulation between SWAT simulations and observations resulted in values of 0.51, 0.62, -6.0 for the streamflow and 0.41, 0.68 and 9.69 % for the nitrate load. Streamflow and nitrate load daily simulations validated for the years between 1990 to 2010 at four selected monitoring stations representing the watershed diversity (Figure 1): Tonneins (outlet, station nr. 20), Portet-sur-Garonne (continental climate, middle part of the watershed, station nr. 6), Roquefort (mountainous climate, upstream, station nr. 4) and Villefranche (Mediterranean climate, upstream, station nr. 14). Both simulated and observed time series show the same tendency (Figure 3 and Figure 4). According to the criteria provided by Moriasi et al. (2015; 2007), the streamflow simulations of the model can be considered as satisfactory over the entire watershed. Concerning nitrate load simulation performances, seven stations including the outlet

(Tonneins, n° 20) show good performances for the three criteria whereas the simulations for other stations performed below the Moriasi et al (2015) standards. However, except for one station (nr. 7, Save River at Larra), all the $R^2$ criteria are evaluated as "satisfactory" for both the calibration and validation periods.

The NNB was computed at daily scale then integrated at monthly and seasonal scale. The NNB estimation was validated by comparing simulations with in situ measurements (Table 1). The comparison was made for the following sampling sites (in $gN.m^{-2}.d^{-1}$): Lézat, Montégut and downstream of Toulouse (Figure 1). The goodness-of-fit of the simulation was determined by using PBIAS and $R^2$.

## 2.6. Ecological Functions Indicators and Statistical Analysis

The investigated ecological functions were related to NR and NP, including in NNB. These functions were analyzed by using the model to simulate both indicators, the NNB and the NNB rate, at the scale of each reach. In the model setup, we defined 1,320 reaches that were around 1.3 and 16.1 km long. The in-stream nitrogen cycle in SWAT is simulated by the QUAL2E module. SWAT model permits to turn off the QUAL2E module that allowed us to distinguish the “physical processes”. The difference between the “physical processes” (when the QUAL2E module is turned off) and the processes when the QUAL2E module is switched on allowed us to distinguish the in-stream biological processes.

### 2.6.1. Nitrate Net Balance (NNB) and Nitrate Net Balance Rate (NNBR)

The indicator of NNB (Eq. 1) is defined as:

$$NNB = \frac{[NO_3\ load\ \text{OUT}] - [NO_3\ load\ \text{IN}]}{\text{Reach wetted area}} \quad \text{(Eq. 1)}$$

NNB is calculated at the reach scale by the in-out nitrate load difference divided by the wetted area. The wetted area is determined by multiplying the wetted perimeter by the length of the reach. The wetted area represents the surface of the cross-sectional area that is “wet” in

273 a reach. The unit of this indicator is $gN.m^{-2}.day^{-1}$. If the value of the NNB is negative, nitrate
274 is removed from the surface water, which indicates the Nitrate Removal (NR). NR describes
275 the amount of nitrate that is retained or withdrawn from the river system either by
276 denitrification, aquatic plant assimilation or sediment retention. If the NNB is positive, it
277 indicates that nitrate inputs are higher than sinks, meaning that Nitrate Production (NP)
278 occurs. In the SWAT model, input nitrate fluxes into the reach come from upstream subbasins
279 drained at this point. The nitrate outputs from upper subbasins are composed of runoff, lateral
280 and aquifer fluxes that are entering the stream.

281 The indicator of NNBR in $m^{-2}$ (Eq. 2) is defined as:

282 $$NNBR = \frac{\Delta NO_3}{[NO_{3\,load}\ \mathrm{IN}]} \quad \text{(Eq. 2)}$$

283 NNBR is a weighting of NNB by nitrate load that enters the reach. This indicator
284 removes the effect of the discharge that has a huge impact on seasonal analysis and allows the
285 comparison of the NR capacity of each reach.

286 2.6.2. Identifying relevant factors for NR

287 A statistical analysis has been carried out to identify influencing factors for NR.
288 R studio software with the packages ade4, MASS, and stats (https://cran.r-project.org) for
289 Principal Component Analysis (PCA) as well as Kruskal Wallis test was used for this analysis.
290 Variables used in the statistical analysis are the slope of the terrain or of the river and the
291 rivers' width, the watershed area, the discharge, and the Froude number. The Froude number
292 is the ratio of mean water velocity and wave velocity, which provides information about the
293 river regime. The calculated Froude number of the river suggests that its regime could be
294 hypocritical (fluvial) (<1) or supercritical (torrential) (>1) indicating the in-stream physical
295 processes behavior. Slopes, watershed area, river slope, and width, as well as Froude number
296 were received by GIS analysis and simulated river discharge. A clustering using the K-means

297 algorithm (Jain, 2010) on these variables was done in order to find a correlation between NR
298 and hydro-morphological factors.

299 ## 3. RESULTS

300 ### 3.1. Assessment of NNB in the Garonne River Network

301 The model simulation is satisfactory with a PBIAS of 5%, a standard error of 0.005 and
302 an $R^2$ of 0.96 between observations and simulations (Table 5). In the flood plain area, two
303 observed flow regimes, 50 and 100 $m^3.\ s^{-1}$, were sampled (Teissier et al., 2008) and the mean
304 PBIAS of NNB between observed and simulated values is evaluated as satisfactory with
305 respectively 10 and -20%. The comparisons between the simulated NNB and the in-situ
306 measurements of Sánchez-Pérez et al. (2009) (sampled at each season) are also satisfactory
307 with a PBIAS of 6%. During winter, NNB is equal to - $7.10^{-4}$ and - $8.10^{-4}$ $gN.m^{-2}.d^{-1}$ for
308 observation and simulation and equal to - 0.059 $gN.m^{-2}.d^{-1}$ and - 0.055 $gN.m^{-2}.d^{-1}$ during
309 spring.

310 Downstream of the Garonne River both indicators (NNB and NNB rate) are positive
311 and show the NP (Figure 5). We can distinguish among two types of reaches: “production”
312 reaches, characterized by high Strahler numbers (superior to Strahler 5, such as the Garonne
313 at Tonneins (outlet)), and "removal" reaches, in the remaining watershed (such as Salat at
314 Roquefort), that shows nitrate retention (Figure 5). In lowland areas, where the slope is
315 median and Strahler numbers are low (these areas are located in the Centre of the watershed),
316 NR is higher than in the rest of the watershed. The highest NR occurred in the intermediate
317 reaches (Figure 5-A), corresponding to reaches between third and fifth Strahler order (67 % of
318 the watershed reaches), whereas the highest removal rate happened in the upper part of the
319 watershed (Figure 5-B). For 82 % of the total watershed, NR is removed by the intermediate
320 reaches. To summarize, in the Garonne River, the hot spots for NR are in the intermediate

321 reaches and for NP, hot spots are located in reaches with high Strahler numbers (higher than
322 fifth Strahler order).

323 ## 3.2. NNB Rate Dynamics

324 ### 3.2.1. Seasonal Variation

325 The comparison of the seasonal monthly average NNB rate variations (Figure 6)
326 shows that the removal rates are higher during summer and spring with median values of
327 $-3.92.10^{-4}$ $m^{-2}$ and $-1.04.10^{-4}$ $m^{-2}$, respectively. Statistically, the NNB rates among seasons are
328 significantly different. The monthly average removal rate is still high during spring (Figure 6)
329 for both physical (river connectivity) and biological processes (water column and benthic
330 processes including in QUAL2E module) (Section 2.3). However, during summer, the
331 biological part produced nitrate with a median value of $+ 3.54.10^{-3}$ $m^{-2}$.

332 During spring, NR is the strongest with 45% of the rivers removing more than
333 - 0. 1 $gN.m^{-2}. d^{-1}$. Monthly average NNB is lowest during winter, about 40 times smaller
334 than NNB during spring. However, the highest NNB variability occurs during spring (±1.77
335 gN
336 $.m^{-2}.d^{-1}$) and winter (±1.45 $gN.m^{-2}.d^{-1}$) whereas the summer and autumn NNB variation are
337 respectively five (±0.36 $gN.m^{-2}. d^{-1}$) and three times (±0.58 $gN.m^{-2}. d^{-1}$) less than winter
338 values. In conclusion, the hot moments of NR seem to occur during summer and spring
339 seasons whereas NP is the most important in winter.

340 ### 3.2.2. Spatio-Temporal Variability of NNB

341 The NNBs in reaches vary along the watershed and among the seasons (Figure 7). The
342 NNB spatial variation, ranging from - 6.27 to + 2.46 $gN.m^{-2}. d^{-1}$, is stronger than the temporal
343 variation (ranging from – 2.56 to + 2.7 $gN.m^{-2}. d^{-1}$). The NNB shows a clear spatial variation
344 during winter and summer (Figure 8): downstream near the outlet the NP occurs during the

345 entire year. The lowland area (Strahler order between 3 and 5) has the highest variability of
346 the NNB rate (Figure7).

347 If we look at the respective contributions of physical and biological processes, the NNB
348 produced by the physical processes is always negative. There is no production of nitrate
349 during the year (Figure 8-C). This NR produced by the physical processes is stronger in the
350 lowland during summer (Figure 8-D) NR of the physical processes is higher in summer (0.36
351 $gN.m^2.d^{-1}$, Figure 8-D) than in winter (0.07 $gN.m^2.d^{-1}$, Figure 8-C). Looking at the NNB due
352 to biological processes (Figure 8-E, Figure 8-F), it can be observed that most of the watershed
353 reaches produce nitrate during summer except for upstream rivers that retained a small
354 amount of nitrate. As expected, during winter with low biological activities, main reaches
355 produce nitrate whereas small reaches retained a low amount of nitrate.

356 ### 3.3. NR and influencing factors

357 #### 3.3.1. Effects of hydro-morphological factors

358 Since NR differs from upstream to downstream, we made a first multivariate analysis
359 to determine which hydro-morphological factors have an impact on NR. The Principal
360 Component Analysis (PCA) shows that NR is correlated to the subbasin slope value,
361 discharge, and Froude number.

362 A cluster analysis groups reaches based on slope, discharge and Froude attributes.
363 Since the slope factor has the strongest weight in the description of NR, the cluster separates
364 the data in three groups of reaches depending on their slope value (Figure 9):

365 - Group 1, named S1, determines rivers with a slope higher or equal than 1%
366 - Group 2, named S2, characterized by a slope between 0.5 and 1%
367 - Group 3, named S3, defined by a slope lower than 0.5%.

368 Over 1320 reaches were defined by the model, 50% of them belong to S1, 29% to S2
369 and 21% to S3. The NNB of physical and biological processes are statistically different
370 between these three groups (Figure 10-B and Figure 10-C), whereas S2 and S3 do not differ if
371 we consider all the processes (Figure 10-A).

372 3.3.2. Background Effect of Nitrate Concentration

373 At the outlet, statistical correlation shows that NR is explained at 30% by nitrate
374 concentrations. However, there is no correlation ($R^2$ = 0.003, p-value>0.05) between these
375 two variables (NR and nitrate concentration) if all reaches of the Garonne watershed are
376 considered. When we focused only on the intermediate reaches (reaches of S3, with slope
377 above 0.5%, between third and fifth Strahler), with the highest NR capacity, a correlation
378 between background nitrate concentration and NR is found ($R^2$= 0.74, p-value<0.001)

379 3.3.3. Land Cover Effect

380 The vicinity of rivers might have an impact on the NNB. Figure 11-A presents the
381 quartiles of inter-annual monthly simulated NNB distribution for each land cover type
382 surrounding the reaches. We considered each land cover of each subbasin and NNB was
383 weighted by the area of each land use in that subbasin. In this study, all land uses are
384 considered to have an impact on in-stream stream processes in rivers. Quartile 1 shows the
385 percentage of reaches removing nitrate the most with NNB less than -0.5 $gN.m^{-2}.d^{-1}$, whereas
386 quartile 4 displays the percentage of reaches producing nitrate (NNB above 0 $gN.m^{-2}.d^{-1}$ ). No
387 quartile 1 and 4 values are found in the bare rock area, which can be explained by the low
388 biological activity in this region. Since this type of area represents only 0.68 % of the
389 watershed, it is considered as not representative. Quartile 4 – containing reaches producing
390 nitrate - are the more frequent in the category “Others”, which incorporates mostly urban and
391 industrial areas (67%). Rivers surrounded by forest (49%), meadow (54%) and agriculture
392 (51%) remove the most nitrate (quartile 1 and 2). Figure 11-B explains the repartition of NNB

depending on crop management according to the Corine Land Cover 2012 classification. Reaches surrounded by planted forest of poplar trees remove nitrate the most (48 %) and do not produce as much nitrate (less than 3 %) as reaches surrounded by pasture (less than 1%).

# 4. DISCUSSION

The discussion focuses on three items: (i) the performance of the model regarding hydrology, nitrate loads and NNB indicator, (ii) the capability of the model to simulate the variation of the NNB in space and time, and finally (iii) the determined influencing factors that can explain the behavior of the NNB in reaches.

## 4.1. Model Performance

The calibration of our model is highly dependent on the variation of the conditions in the different spatiotemporal pedo-climatic zones as well as on discharge variability, agricultural practices, and dam management in the Garonne watershed. In our case, the simulation results on streamflow, nitrate load, and NNB can be compared with a few studies in the case study area.

### 4.1.1. Hydrology

Simulated water yield (around 350 $mm.yr^{-1}$) is in the same range as described by Tockner et al. (2009), and evapotranspiration (450 $mm.yr^{-1}$) is in agreement with Grusson et al. (2018). The streamflow at station Tonneins varies between 513 to 623 $m^3.s^{-1}$ in the period of 1990 to 2010 (Espitalier-Noël et al., 2016; Grusson et al., 2018) which is in the same range as in our study (561 $m^3.s^{-1}$). According to Moriasi et al. (2015), our daily discharge simulations can be evaluated as good to very good (Table 3). The poorest streamflow performances are achieved at the gauges of some rivers that are known to be subject to significant human stressors (urban effluents, dams, and agricultural managements), such as the Save River at Larra (Boithias et al., 2014).

To compensate for the lack of data of other hydrological components such as ground and sub-surface water and to avoid equifinality issues, this study tries to use other values such as crop yields and irrigation volumes to provide a number of the water use partitioning and nitrogen inputs. The achieved performance metrics for the calibration and validation periods as well as the comparison with other studies confirm the suitability of the SWAT model to simulate realistically streamflow, irrigation volume, and crop yield.

#### 4.1.2. Nitrate loads

Simulated nitrate loads of this study (1 478 $kgN.km^{-2}. y^{-1}$) are similar to others reported in the literature, with ranges between 900 to 1500 $kgN.km^{-2}. y^{-1}$ (Sutton et al., 2011). Due to confidentiality laws and resulting lack of data on spatially explicit fertilization rates, the assessment of the effects of agricultural management strategies at the micro-scale (farm level) is nearly impossible (Volk et al., 2008). Hence, nitrogen inputs by agriculture and municipalities have to be generalized. However, these simplifications enable a more or less satisfactory simulation of nitrate losses on a larger scale. For example, downstream Toulouse, the average concentration of nitrate observed is 3.4 mg. $L^{-1}$, which is due to the effect of the wastewater treatment plant effluents, whereas the simulated concentration based on simplified effluents is only 2.6 $mg.L^{-1}$ (PBIAS of 22%). Similarly, Jégo et al. (2008) showed that nitrogen leaching for Sugar beet crop is equal to 20 $kgN.ha^{-1}$ whereas our study simulated leaching of 18.5 $kgN.ha^{-1}$ which is in the range of the uncertainties of crop management information and N application periods (Jégo et al., 2008).

In general, the lack of both long term series of daily water quality data and high-density networks of water quality monitoring stations has limited our capacity to evaluate the simulations, which represents a general problem and results in uncertainties. Despite the reported uncertainties, the performance of the simulated nitrate loads is considered

442 satisfactory for the majority of the watershed (according to the suggestions of Moriasi et al.
443 (2015)).
444
445 4.1.3. Nitrate Net Balance (NNB)
446 The validation such as streamflow, nitrate load in different points in the river network,
447 crop yields, and irrigated volume enabled a good representation of the nitrate transfer and so
448 the NNB. Moreover, the NNB simulated in SWAT is validated by in-situ measurements (Table
449 5). Knowing that each comparison has its uncertainties, the validations of multiple variables
450 support a satisfactory representation of the watershed processes and their ecological functions.
451 All NNB ranges that are not represented by in-situ measurements have been validated with
452 this methodology ("indirect validation"). Due to the high uncertainties of the daily NNB
453 simulations, monthly values for the comparison of the simulation and observation values are
454 used. This procedure is also more suitable for comparing orders of magnitudes of NNB. In our
455 study, the simulated maximum daily amount of nitrate removed from the corresponding reach
456 (-0.12 $gN.m^{-2}. d^{-1}$ with a standard deviation of 0.06) is in the same range as reported in the
457 study by Sauvage et al. (2018) (-0.19 $gN.m^{-2}. d^{-1}$ with a standard deviation of 0.07). Some
458 studies using microcosm tried to evaluate the NNB of the biological processes, which range
459 between -3.51 and -0.01 $gN.m^{-2}. d^{-1}$ in Yao et al. (2017) and from –0.089 to -0.031 $gN.m^{-2}. d^{-1}$
460 in Liu et al. (2017), which is in the range of our results (from –6.28 to 2.46 $gN.m^{-2}. d^{-1}$). This
461 study shows that even with a basic model depending on algal biomass and dissolved oxygen
462 such as QUAL2E coupled to SWAT (Neitsch et al., 2011) can represent the ecological
463 function on a large scale. This module describes processes such as algae uptake,
464 mineralization, nitrification, and ammonification. Recently, other models developed for fine-
465 scale, such as RiverStrahler, are more comprehensive for the in-stream biological processes
466 description than the SWAT module used in this study.

Considering physical and biological processes separately, the ranges are completely different. The physical effect on NR is mainly explained by a mixture of waters from different sources and associated with nitrate loads. During summer and winter, as the Garonne watershed has a rain and snowfall regime, we have 2 low water periods, the exchanges between groundwater and water columns are characterized by low discharge and low runoff, and thus higher via the hyporheic zone (Marmonier et al., 2012). Hence, the main contribution is coming from groundwater flow. During this low water period, the nitrate loads are driven by physical processes and come from low land areas and floodplains where significant shallow groundwater aquifers can be found, which are mainly polluted by agricultural activities. This shallow groundwater is mainly polluted by agricultural activities (Jégo et al., 2008; Sánchez Pérez et al., 2003). During the autumn and the spring, lateral subsurface flow, as well as surface runoff, contributed to the river nitrate load (Bernard-Jannin et al., 2017; Peyrard et al., 2011; Weng et al., 2003). The biological processes simulated by SWAT have a much lower influence on the overall NR than the river connectivity (physical processes). The proportion of biological and physical processes in the total NNB is 15 % to 85 % in summer, 23 % to 77 % in fall, and 21 % to 79 % in spring and winter. During some season and flood events, biological and physical processes compensate for each other. For example, downstream of Toulouse, during spring, nitrate is produced by the biological part (12.2 $gN.m^{-2}.d^{-1}$) whereas the physical part removes 14.7 $gN.m^{-2}.d^{-1}$ of nitrates. Considering the balance of physical and biological processes, downstream of Toulouse, the river removes 2.5 $gN.m^{-2}.d^{-1}$ of nitrates. In some reaches (generally upstream), only biological processes occur whereas in other parts physical processes are the main drivers. Both of these processes depend on transient storage in the reach. If the transient storage is high enough for biological processes to be involved, biological processes could be more important than the water connectivity effect. It could be interesting to focus on these different types of reaches to find a pattern of processes.

493 By comparing our results with the finding of Teissier et al. (2008) (representing only
494 benthic processes), it becomes clear that the model is close to the amount of the NNB found
495 in the biofilm and sediment compartments. The biological module of SWAT satisfactory
496 simulates the interactions between sinks and sources that occur in the river. Quantifying the
497 transformation of nitrogen into nitrate in the rivers is necessary to develop cost-efficient
498 management strategies to reduce nitrate pollution and increase the water purification
499 efficiency.

500 Quantification of nitrogen by using QUAL2E has been approved in many other studies
501 (Little and Williams, 1992; Migliaccio et al., 2007; Ryu et al., 2016; Salvetti et al., 2008).
502 QUAL2E is very well suited for waste load allocation studies and management decision
503 planning(Brown and Barnwell, 1987). The model is widely used in research studies and
504 commonly applied as a standard model in water quality projects to evaluate other models
505 (Shanahan et al., 1998). However; QUAL2E is specifically intended for the steady-
506 streamflow in the water quality regulations and there is some criticism on the use of QUAL2E
507 on watersheds with high variations in streamflow and high fluctuations over a diurnal and
508 shorter time period. Nonpoint sources of pollutants to the river are highly driven by rainfall
509 and vary significantly over time. These variations may deviate significantly from the
510 underlying assumptions of QUAL2E (Shanahan et al., 1998). In our study, the objective is to
511 determine the overall trend in average algal production in the Garonne watershed area that is
512 important in the uptake of nitrate. Thus, working on a monthly scale avoids large flow
513 variations and enables staying within the validity range of QUAL2E. Furthermore, we have
514 been able to validate the SWAT-QUAL2E simulation by comparing the model outputs with
515 some other studies (Liu et al., 2017; Tisseuil et al., 2008; Yao et al., 2017a). In the case of the
516 Garonne watershed, NR is due to denitrification and algae uptake. However, some studies
517 show that the denitrification is offsetting during day and night in Garonne watershed (Tisseuil
518 et al., 2008). The model is taking into account mainly the uptake of nitrate by biomass in the

519 water and on the surface of the sediment. The model also considers that denitrification is very
520 low. So, by running the model at monthly step we undergo the sub-daily processes
521 (sink/release of nitrates like denitrification and plant uptake/mineralization) and keep the main
522 processes represented by quality model. However, at an intra-daily scale, the model user
523 might consider another water quality model integrating both the sediment/surface water
524 interface and all the in-stream biomass, such as the RiverStrahler model (Billen et al., 1994),
525 in order to analyze the weight of each compartment (denitrification, biofilm uptake, algae
526 uptake…).
527

528 ## 4.2. NNB Variations in Time and Space

529 ### 4.2.1. Hot Spots And Hot Moments Of NR

530 Our method highlights hot spots, hot moments and also some explanatory factors of
531 nitrate retention that are easy to measure. NNB includes two functions: NP and NR. Spatial
532 and temporal variations affect both functions (Figure 5, Figure 6 and Figure8). NP occurs
533 mostly during winter, when in-stream processes are the least active, and in major rivers,
534 downstream of important cities (Figure 5-A). NR occurs mostly during summer and in the
535 intermediate rivers (between third and fifth Strahler order and a slope above 0.5%). If we look
536 at the patterns between biological and physical processes (by dilution, mainly) (Figure 6),
537 season variability is driven by physical processes whereas there is no significant difference
538 between NNB due to biological processes.

539 Biological activity and hydrologic variability influence nitrate sources and sinks
540 through seasons and across the watershed. The hypothesis that the variability is influenced by
541 other factors such as biodiversity is confirmed by Sauvage et al. (2018) and Yao et al. (2017).
542 However, the amount of nitrate removed and the rate of this removed nitrate is different
543 because of the variability of some environmental conditions. Some rivers remove an

important quantity of nitrate, but this amount is influenced by the input of nitrates and by the size of the river as well as by the wetted area. The active biological processes occur in the benthic and pelagic parts of the river as well as the hyporheic zone where most nitrate is removed (Marmonier et al., 2012; Sauvage et al., 2018). The wetted area of a reach is variable in time and space (Alexander et al., 2009) and can be an important factor affecting the variability of NR both temporally and spatially.

Reaches in an agricultural area seem to retain more nitrate (Figure 11-A) and their NNB variabilities during the year are higher than reaches surrounded by other land use, especially in the intermediate rivers (Figure 7). However, the NNB rate (Figure 5-B) shows a lower spatial variability than NNB (Figure 5-A) that is influenced by environmental factors such as the reach characteristics. Nitrate concentration seems to have an influence, but it does not explain NR directly if we consider all reaches. However, for intermediate reaches (between third and fifth Strahler order and a slope above 0.5%), NR could be partially attributed to nitrate background concentration. Nitrate concentrations in the river have a background effect on NR so it should be directly or indirectly taken into account in further studies. On top of that, our statistical analysis shows that NR may be explained mostly by hydro-morphological factors.

Reaches removing nitrate the most are concentrated in S3 (Figure 10). They are characterized by plain areas with low slopes (<0.5%). S3 (Figure 10-A) integrates both the hot spots of NR (the intermediate reaches) and NP (reaches with high Strahler number). Although production is observed in the high Strahler reaches as shown in the upper whisker of S3 of Figure 10-A. S3 seems to be the group with the most activities, such as high NR caused by physical processes (Figure 10-B) as well as high NR and NP caused by biological processes (Figure 10-C). S3 comprises the intermediate reaches and high Strahler number reaches where hillsides leach more nitrogen because of the higher density of stressors such as agricultural practices and municipalities' effluents. We can conclude that small rivers (Strahler order under

570 five) in the flat areas of the Central watershed are the most actives reaches. In these special
571 cases of intermediate small streams, low slopes are correlated with low discharge (Figure 9),
572 which causes a longer residence time and more interactions between the water column and
573 sediment. This may facilitate NR processes (Zarnetske et al., 2011). In fact, biological
574 activities (i.e., biofilm in the sediment) are the biggest nitrate consumers, especially under low
575 oxygen conditions in river sediments that trigger denitrification (García-ruiz et al., 1998). This
576 removal may have happened during the aquifer flows in the hyporheic zone where oxygen is
577 lower than in free water. The nutrient spiraling phenomena in the fluvial continuum might
578 explain this removal (Vannote et al., 1980). In the headwaters, organic matter degradation is
579 accelerated by some organisms whereas downstream microbial activity and algae density are
580 stronger, thereby controlling NR in this part of the watershed (Sauvage et al., 2018).

581 Spring and summer are the seasons where the removal rate is higher (Figure 6). These
582 seasons are the period where flora and fauna are the most active (McClain et al., 2003).
583 Moreover, these seasons are characterized by low-flow periods where streams export less than
584 50% of inorganic nitrogen input (Peterson et al., 2001). If we consider all processes, NR
585 occurs mostly during spring and summer, but the highest NNB variability occurs during
586 spring and winter when nitrate is removed especially from physical processes and nitrate is
587 produced mostly by biological processes. Spring and winter are flooding seasons that can
588 have an impact on the dilution effect and biodiversity activities (Rolls and Bond, 2017).
589 During high flows, in-stream processes and the fluxes associated with NR or NP still exist,
590 but these fluxes are very low compared to total nitrate fluxes. Hence, during high flow, NR
591 and NP are masked when we focused only on the balance. Moreover, Richardson et al. (2004)
592 showed that during high flows the effect of floodplains and backwater areas becomes more
593 important. The NR is efficient when the discharge is low and nitrate concentration is high.

594 4.2.2. Influence of hillside characteristics on NR

595 Understanding the NR capacity and its behavior under stressors (any external factor
596 derived from human intervention as municipalities' effluents, land management) (Sabater et
597 al., 2019) in the aquatic system is important for analyzing impacts of nitrogen on water
598 quality and targeting remediation measures to protect our water resources (Grizzetti et al.,
599 2016). Our results have shown that the hillside land cover has an impact on in-stream NR
600 (Figure 11). The correlation between hillside land cover and in-stream NRs might be
601 explained by the impact of hillside land cover on the nitrate concentrations in the surface
602 water by leaching.

603 When an ecosystem is subjected to human disturbance, NR can evolve significantly
604 and sometimes irreversibly (Chapin Iii et al., 2000). This study shows that human activity
605 land use might have an impact on this function. NR occurs mostly in the forest areas, which is
606 explained by the denitrification activated by nitrate inputs, soil biodiversity, and the forests'
607 soil moisture (Larson et al., 2019), but surprisingly also in the agriculture area. Moreover, the
608 analysis of the type of agriculture shows that planted forests remove nitrate the most (Figure
609 11-B). In this study, we considera plantation forestry system as a forest managed for
610 production purposes (Carle and Holmgren, 2003). This type of plantation undergoes tree-
611 cutting that increases the plant nitrogen uptake compared to senescent trees (Fukuzawa et al.,
612 2006). Moreover, the fertilization of the planted forest will provide some nitrogen inputs
613 controlled by tree roots that avoid an important nitrate runoff (Udawatta et al., 2017) that
614 permits the removal processes to be activated.

615 The indicator of NNB is influenced by climate, nitrate effluents from natural processes or
616 human activities and geomorphological characteristics. However, it is interesting to explore
617 factors that can explain and forecast NNB evolution.

618 NP and NR functions variability is not only impacted by the upstream-downstream and
619 seasonal variation but also by agriculture and settlements with non-point and point-source
620 pollution which explain the mean nitrate concentrations in the reach. Due to more human

621 activities and more pollutions, NP frequently occurs downstream of municipalities. In
622 conclusion, it becomes clear that specific landscape elements, such as hillsides, riparian
623 zones, and floodplains, have an impact on in-stream processes and more precisely on
624 ecological functions, strengthening the idea that further studies should focus on the integration
625 of both land and in-stream processes for ecological functions assessment.

626 ### 4.3. Influencing factors for in-stream NR

627 River morphology and the hydrological regime have an influence on NR in our study area,
628 as it was shown with hydro-morphological factors (discharge, Froude number, slope) and NR.
629 The initial hypothesis was that the nitrate concentration is a driver of the NR rate
630 (Sánchez-Pérez et al., 2009). In this study, the correlation between nitrate concentration and
631 NR across the Garonne watershed is not obvious except for intermediate reaches (between
632 third and fifth Strahler order and a slope above 0.5%) and could be explained by the small
633 range of nitrate. The relationship between NR and influencing factors includes background
634 nitrate concentration. Without in-stream nitrate or low nitrate concentration, NR cannot occur
635 or proceed with higher capacity even if the "potential removal capacity" of the reach is high.
636 This is not appropriate for the contrary case: high concentrations of nitrate are not correlated
637 with a high NR potential. In our case, there is a positive relationship between nitrate
638 concentration and the removal rate. The ecological tipping point (Dai et al., 2012), where the
639 NR would collapse with more nitrate concentration is not confirmed in our study. However, it
640 has to be pointed out that the current SWAT model would not be able to simulate it in a
641 sufficient way. Hence, this study encourages further work to develop nitrogen cycle modeling
642 for improved inclusion of the ecological tipping point.
643 Another hypothesis, formulated by Alexander et al. (2009), was that river size and
644 reach's Strahler number, which are indirectly correlated with the slope of the reach, have an
645 impact on the NNB. In the mountains, sloped sites are less affected by human use than

lowlands. This hypothesis leads to the assumption that in upstream reaches (where human influences, such as channelized rivers, are less intense) the water quality regulation service may be underestimated (because of the lack of nitrates).

Our results on the impact of the slope, nitrate concentration, Froude number, and discharge on NNB suggest further investigations of the relationship between these factors, in order to consider these factors as indicators of NNB. By demonstrating that the biophysical diversity controls the function of NR, Sauvage et al. (2018) develop the idea of the relationship between hydro-morphological factors and NNB. The functional compartments existing in a reach (Sauvage et al., 2018) act on the nitrate uptake or release capacity of the river, and also, hydro-morphological factors that influence NR efficiency. Our results suggest a classification in three groups: a) upstream river section with gravels, low water level, and high velocity, the main functional compartment is epilithic biofilm; b) middle part of the river-dominated mainly by gravels, hyporheic zone and greater interactions with groundwater, and c) the lower river section that is characterized by fine sediments, high water levels, and low velocity. This classification is confirmed by observing the same slope and other hydro-morphological characteristics. NR can be divided into three groups depending on slopes, river discharge, and Froude's number. These groups can be distinguished by slopes and nitrate process interactions. An important finding is here that there is a synergy between river structure and function to explain NR. Moreover, NR is mainly impacted by the biofilm in the hyporheic zone by the influence of macroinvertebrates (Liu et al., 2017; Sauvage et al., 2018; Yao et al., 2017). Considering both river structures and biological activities in future studies could be a way to better understand ecological functions without going through a complex model. This study wants to encourage researchers to develop studies in that field.

## 5. Conclusion

This study analyzed for the first time space and time dynamics of the Nitrate Net Balance (NNB), an indicator of water regulation service, under multiple human stressors in a large

watershed. These dynamics were investigated at the reach scale and monthly time step to characterize the "hot spots" and "hot moments" related to Nitrate Removal (NR) and Nitrate Production (NP) by using both modeling and different in-situ datasets. The model has been calibrated and validated on different variables linked to human stressors and natural processes (crop yield, irrigation volume, streamflow, nitrate load). This calibration-validation procedure enabled an indirect calibration of the NNB. Hot spots, intermediate reaches of the watershed (between third and fifth Strahler order and a slope above 0.5%), and hot moments, summer and spring, of NR were given in evidence. Finally, this study identified drivers controlling the dynamic of NR process. According to our statistical analysis, NR is negatively dependent on the slope except for rivers with high Strahler number. The second part of the analysis highlighted the influence of river hydro-morphological characteristics and land use on NNB. Considering the NNB and physical factors, some such as discharge, slope and Froude number, influence the NR in all reaches of the Garonne watershed. Finding a relationship between these variables, river hydro-morphological characteristics and land management could be a good way to help stakeholders in water management decisions and boost awareness and involvement of people for sustainable management of water resources. Wetlands and riparian areas are hot spots of NR and understanding their influence on NR balance could give us some insights about management decisions.

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# Support Vector and Locally Weighted regressions to monitor monoclonal antibody glycosylation during CHO cell culture processes, an enhanced alternative to Partial Least Squares regression.

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**Abstract**

Since monoclonal antibodies (mAb) are sensitive to the manufacturing process, several mAb variants can be the result of a single batch production. The most critical source of heterogeneity is glycosylation which has a profound impact on safety and efficacy of the final product. Implementation of monitoring and control of the process using the Quality by Design (QbD) approach may help to ensure mAb specifications, although its implementation is limited by the availability of real-time specific measurements. All current approaches to elucidate mAb glycoforms require sampling and labour-intensive efforts. Thus, glycosylation analysis is often performed with the objective of detecting quality defects at the end of the culture process. In this work, the capability of Near Infrared spectroscopy and chemometric treatment to accurately monitor mAb glycosylation during CHO cells cultures using *in situ* probes is shown for the first time. Real-time monitoring of glycosylation, in terms of high mannose isoforms, fucosylated, sialylated and galactosylated isoforms as well as non-glycosylated mAb, has been successfully performed by the novel use of Locally Weighted Regression (LWR) and Support Vector Regression (SVR). These encouraging results open the way for the implementation of control systems on the impact of cell culture operating parameters on mAb heterogeneity, particularly glycosylation, during CHO cell culture processes through the QbD approach.

**Keywords:**

Antibody real-time glycosylation monitoring, support vector regression, locally weighted regression, Process Analytical Technology, Quality by Design

## 1. Introduction

Monoclonal antibodies (mAb) produced in animal cell culture processes represent a success in terms of clinical benefit for patients and revenue generated by biopharmaceutical industries. Such molecules are quite sensitive to changes in manufacturing processes and thus several mAb variants could be produced within a single batch due to post-translational modifications. Glycosylation is the main source of mAb variability which can strongly impact mAb clinical properties. Therefore, the control of glycosylation specific profiles of mAb during the process is critical for therapeutic efficacy and patient safety. For this purpose, regulatory agencies proposed the Process Analytical Technology (PAT) strategy to control pharmaceutical manufacturing processes through the continuous adjustment of Critical Process Parameters (CPP) which affect Critical Quality Attributes (CQA) of the product. Accordingly, continuous monitoring of CQA, such as mAb glycosylation, is required to establish advanced retro-control systems to guarantee mAb specifications [1].

However, continuous monitoring of mAb glycosylation is challenging since it requires analyses at a relatively high cost. Complete mAb glycosylation analysis must include identification of sugar chains attached to glycosylation sites (macro-heterogeneity) and of the sugars moieties within the glycan chain (micro-heterogeneity). Indeed, mAb macro- and micro-heterogeneity analyses involve several steps, such as enzymatic digestion, labelling, derivatization and separation, followed by structural analysis, usually using mass spectrometry (MS) [2]. The complexity of such analyses implies significant delays, 5 hours to 2 days, mainly for the acquisition of process information, thus mAb quality analysis is usually performed at the end of the cell culture process [3].

In recent years, intensive efforts have been made to establish PAT as the mean to monitor mAb glycosylation during cell cultures, particularly using automatic at-line or on-line traditional biochemistry approaches with the aim of reducing analysis times and sample

77 volumes. Such approaches allow the presence of mAb glycoforms to be known in a question
78 of hours [4–7]. In the last few decades, vibrational spectroscopy, in combination with
79 multivariate analysis, has been proven to be a promising tool [8], particularly for cell culture
80 monitoring [9]. However, it is mainly restricted to some usual cell substrates and/or by-
81 products such as glucose, lactate or glutamine [10–15]. More recently, a study showed the
82 possibility of monitoring mAb concentration by using either Raman or NIR spectroscopy in
83 real-time during CHO cell cultures [16]. Although Raman spectroscopy led to a slightly better
84 estimation for mAb concentration, NIR spectroscopy showed a higher signal-to-noise ratio,
85 though in more complex spectra. The inferior capacity of the NIR model was thus mainly
86 attributed to the lack of linear PLS regression for handling complex NIR spectra, likely
87 containing information in non-linear ways [16]. However, as far as can be ascertained,
88 glycosylation micro-heterogeneity monitoring has not been addressed. In this study, we
89 showed that *in situ* NIR spectroscopy can be applied for in-line monitoring of mAb
90 glycosylation micro-heterogeneity.
91 As alterations in mAb glycoform patterns may result in strong changes in clinical profiles,
92 manufacturers must guarantee glycosylation specifications to ensure reproducible and
93 consistent clinical performance. The occurrence of several factors that may compromise
94 conservation of clinical profiles is not uncommon in the pharmaceutical industry [17], and so
95 monitoring and control systems are required to ensure mAb properties. Conservation of such
96 properties is due to proper combination of glycoforms with different sugar moieties within the
97 glycan chain. Indeed, it has been widely reported that the presence of fucose, galactose and
98 sialic acid strongly affect antibody dependent cellular cytotoxicity (ADCC), complement
99 dependent cellular cytotoxicity (CDC) and immune modulation of the mAb, respectively [18].
100 On the other hand, high mannose glycoforms are reported to reduce serum half-life [19]. In
101 this context, producing mAb glycoforms with resulting clinical effects similar to those of the

reference mAb is critical for batch approval [17]. In this study, it was shown that NIR spectra can exhibit an estimated correlation to non-glycosylated mAb and total mAb concentration (mAb macro-heterogeneity), as well as to glycoforms containing fucose, galactose and high-mannose structures, including sialic acid within the glycan chains (mAb micro-heterogeneity). Furthermore, this approach could have an immediate application using a NIR spectrophotometer in at-line or off-line modes, which could provide mAb glycosylation information in question of minutes.

## 2. Theory

Spectra, particularly from NIR in-line analysers, are complex since both physical and chemical information is contained, usually in a highly collinearity way. Thus, multivariate calibration particularly for regression, is needed for correlating complex spectra to desired quality attributes or analyte concentration. Though many regression methods are available for building calibration models, as far as it can be ascertained, only Partial Least Squares Regression (PLSR) has been addressed in cell culture monitoring. In this work we analysed the adequateness of PLSR for cell culture monitoring and explored the performance of other regression methods such as Support Vector Regression (SVR) and Locally Weighted Regression (LWR). The intuitive concept of models as well as their characteristics for regression in cell cultures are discussed.

### *2.1 Partial Least Squares Regression (PLSR)*

The PLSR method is based in a reduction variable process in order to treat collinearity. Firstly the spectra (X) and the concentration (Y) matrix is decomposed as [20]:

$$X = TP^T + R_1 \quad (1)$$

$$Y = UQ^T + R_2 \quad (2)$$

where X and Y are spectra and concentration matrices respectively, T and U are the pseudo-scores matrices, P and Q are the pseudo-loadings matrices and $R_1$ and $R_2$ are the residuals

matrices. Matrix decomposition of X and Y matrices are not independent, thus an internal relationship between the scores of X and Y are generated accordingly:

$$U = BT \quad (3)$$

where U is the pseudo-scores of Y to be calculated, T the pseudo-scores of X and B the regressor matrix. PLS works with the constraint that these components explain as much as possible of the covariance between X and Y. Once the regressor matrix has been determined, calculation of y-concentration value from problem sample may be calculated as:

$$Y = T^{*}BQ^{T} + R_1 \quad (4)$$

Where T* is the pseudo-scores matrix of the problem sample, B the regressor matrix, $Q^T$ the pseudo-loading matrix of the model and $R_1$ the residual matrix. Regression based on PLSR offers a relatively simple frame for analysing the relationship between spectral response and prediction by the model. However, it is particularly sensitive to scattering effects and may not properly handle information contained in a non-linear way. Therefore, for building quantitative calibration models using NIR spectroscopy, a common assumption has been focusing on chemical information and limiting the contribution of physical data contained in spectra by the use of spectral pre-treatment [21]. However, manipulation of spectra for limiting scattering effects may also disturb chemical information [22] and thus a compromise must be adopted.

PLSR is a variable space-based regression method which calculates the relationship between each of the variables (absorptions at different wavelengths) and compound concentrations. Therefore, such a relationship should be relatively constant during the whole culture process in order to maintain accurate estimations. Though it is an obvious assumption, caution must be taken considering the strong physical and chemical variations of culture media during cell culture progression. Deviations from this assumption are usually observed as non-linear effects (Figure 1-A) and may limit the predictive capability of models. In such cases, the

152 regression equation leads to a linear trajectory of predicted values (grey dotted arrow in
153 Figure 1-A), limiting prediction on non-linear sections (cross in Figure 1-A). As far as can be
154 ascertained, this fact has not been addressed yet in cell culture monitoring. Therefore, the
155 novel use of space-based regression methods which firstly focus on affinity and dissimilarity
156 between samples (culture progression information), and secondly on the relationship between
157 variables (absorptions at different wavelengths), has been evaluated.

158 *2.2 Locally Weighted Regression (LWR)*

159 LWR is mainly addressed for modelling complex relationships for which no theorical model
160 may exist. In contrast to PLSR which generates a regression function considering all
161 calibration points, LWR firstly compares the sample to be predicted with samples within the
162 calibration set. Then only those calibration samples similar to the sample to be predicted are
163 used (the local area) (black circle in Figure 1-A). Then each point of the local area is weighted
164 according to its distance from the sample to predict: close points are given more importance
165 or weight, far points are given less weight; then a regression function of the independent
166 variables is generated employing the weights and in the local area [23] (Figure 1-B).
167 Generation of accurate models then requires adjustment of key parameters such as similarity
168 between samples, definition of the local area and the weights, and the nature of the regression
169 itself.

170 Once spectra have been mapped into a chemometric space (principal component space, latent
171 variable space, among others), the local area is determined by a distance function and
172 specified limit. Since distance as a delimiting criterion may be inappropriate when lacking
173 vast calibration samples in a wide calibration space, several authors have employed distance
174 criteria in terms of near calibration samples [24,25]. Then local calibration samples are
175 weighted according to a weight function, such as the tricubic function (5). Then the weight for
176 a calibration sample is calculated as:

$$W(u) = \begin{cases} (1-u^3)^3 & if\ u \leq 1 \\ 0 & if\ u > 1 \end{cases} \quad (5)$$

$$w_i(x_j) = W\left(\frac{\delta(x_j, x_i)}{d(x_j)}\right) \quad (6)$$

where

$(x_j, x_i)$: Distance between prediction sample j and calibration sample i

$d(x_i)$: Maximum distance involved in each regression

As could be noted, the weights will be large (close to 1) for $x_i$ close to $x_j$, and small (close to 0) for $x_i$ far from $x_j$. Once the region and weights have been determined, regression function in the local region is generated generally using weighted PLSR. Finally, the concentration value for $x_j$ is calculated using the local weighted PLSR regressor (grey dotted arrow in Figure 1-B). In contrast to global PLSR that treats all the regression surface at the same time, as either linear or non-linear, LWR models non-linear regions without compromising linear regions. This approach is particularly adequate for animal cell culture processes in which linear and non-linear behaviour may arise differently during different phases of cell cultures. Moreover, the use of similar samples in the local area could lead to better spectra pre-treatment and thus limiting the loss of information by attenuation of scattering effects in spectra. Drawbacks of LWR are the need of dense calibration samples, vulnerability to outliers [26] and the lack of a mechanistic model where fitted parameters specify particular physical or chemical properties of the cell culture. This is of great concern since regulatory agencies demand that NIRS signals be directly attributed to analytes or be an indirect measurement correlated with light scattering effects [27,28]. Then submission of monitoring procedures would eventually require efforts considering all possible combinations of local regressions.

### *2.3 Support Vector Regression (SVR)*

A relatively novel alternative for non-linear modelling of NIR spectra is SVR [29]. The main difference of SVR from other typical regression methods is that its objective is not merely to reduce the fitting error but to fit the error within a particular threshold (±ε). Then the goal of SVR is to generate a regression function, or hyper plane, that has a maximum number of calibration samples at most an ε deviation from an actual concentration ($y_i$), and at the same time keeping the function as flat as possible [30]. For instance, the hyper plane is considered as:

$$f(x) = (wx) + b \quad (7)$$

$with \quad w \in X, \quad b \in \mathbb{R}, \quad$ and x being a variable related to spectra

Flatness is then assured by minimisation of w, for example minimising the norm as a convex optimization problem:

$$minimise \ \frac{1}{2}|w|^2 \quad (8)$$

Subject to

$$y_i - (wx) - b \leq \varepsilon \quad and \quad (wx) + b - y_i \leq \varepsilon \quad (9)$$

However, it may not be the case that *f(x)*, which approximates all pairs ($x_i$, $y_i$) with ε precision, actually exists. Then a soft margin of slack variables $\xi_i$, $\xi^*_i$, are introduced for coping with unfeasible constraints of optimization (8) as stated by Vapnik [31]:

$$minimise \ \frac{1}{2}|w|^2 + C\sum_{i=1}^{l}(\xi_i + \xi_i^*) \quad (9)$$

subject to

$$y_i - (wx) - b \leq \varepsilon + \xi_i \ , \quad (wx) + b - y_i \leq \varepsilon + \xi_i^* \quad \text{and} \quad \xi_i.\xi_i^* \geq 0 \quad (10)$$

The constant $C$ determines the compensation between the flatness of $f(x)$ and the amount up to which deviations larger than $\varepsilon$ are tolerated. This general procedure is depicted in Figure 1-C. As could be observed, SVR is then less vulnerable to outliers since it could properly generalise and leave the outliers in the soft margin (forbidden symbol in Figure 1-C). In complex multivariate data optimization, (9) can be solved more easily in its dual formulation, which provides the possibility for extending the procedure to non-linear functions. This could be achieved by mapping the $x_i$ patterns into some feature space $F$ [32]:

$$\varphi: x \to F \quad (11)$$

Then standard SVR procedure is applied. Mapping into a higher, linear or non-linear, dimensional space, may require exacerbated computational power, thus the majority of SVR use implicit mapping by kernels. The most common are linear, polynomial and Gaussian radial basis function (RBF) kernels. The nature of the calibration set must be considered for properly selecting the kernel [33]. The linear kernel is useful in large sparse data vectors with linear regularization, the polynomial may fit some soft non-linearity and RBF are general-purpose that are generally applied in strong non-linear regularization or in the absence of prior knowledge [33]. This approach could be used for generalizing difficult to fit data in complex systems. As well as for LWR, monitoring procedure submission could be likely cumbersome for relating SVR parameters to specific chemical or physical properties of the cell culture, particularly in strong non-linear processes mapped into high dimensional feature space.

## 3. Materials and methods

### *3.1 Cell cultures for NIR spectra acquisition*

The bioreactor data set was designed with routinary monitoring for batch culture in mind. Several cultures of CHO cells were performed in 2 L bench-top bioreactors (Pierre Guérin, France) with a 1.5 L working volume: three batch cultures, two feed-harvest cultures with medium renewal and one batch culture with glucose spiking. The three batch cultures were

intended to observe inter-batch heterogeneity as well as in-line and off-line expected routinary responses. Feed-harvest cultures were used for increasing the variance of mAb glycoforms within the calibration process, which could enhance model prediction capability. These were started after a first phase in batch mode, then 2/3 of cell culture was withdrawn and replaced by fresh culture medium. This procedure was repeated 2 and 4 times for these 2 feed-harvest cultures respectively. As relative abundance of particular mAb glycoforms is partially a function of cell culture progression, the use of feed-harvest cultures favoured not only samples with mAb at the beginning of the cultures, but also cell cultures with a wider variability of mAb glycoforms. Batch culture with glucose spiking was used for increasing mAb concentrations so that these values during routinely batch culture monitoring relied preferably within an appropriate concentration range.

The culture medium was a protein-free medium mixture consisting of a 1:1 volume ratio of PF-CHO (HyClone) and CD-CHO (Fisher Scientific) supplemented with 4 mM L-glutamine (Sigma Aldrich) and 0.1% pluronic F-68 (Sigma Aldrich). The genetically modified DG44 CHO cell line was used (human anti-Rhesus D mAb-producing CHO M250-9), kindly provided by Bioprocessing Technology Institute (Singapore). Dissolved oxygen (DO) was controlled at 50% air saturation and agitation rate was fixed at 90 rpm throughout the culture. Temperature was maintained at 37 °C and pH was set and controlled at 7.2 using 0.5 M sodium hydroxide and $CO_2$.

*In-situ* (or in-line, invasive) spectral scanning of bioprocess culture media was carried out with a NIR transflectance probe with 1 mm pathlength (Precision Sensing Devices, MA). The autoclavable probe was connected to an Antaris II spectrometer (Thermo Scientific, USA). Each NIR spectrum corresponded to an average of 128 scans from 1,000 to 2,500 nm.

*3.2 Off-line analyses*

Off-line concentration of total mAb was determined using an enzymatic kit (Roche Life Science) with an automatic spectrophotometer (Thermo Scientific GALLERY) against external standards. The nature and concentration of mAb heterogeneity in the form of glycoforms was elucidated by HPLC/UHPLC-mass spectroscopy analysis, as previously described [15]. Off-line concentration values for calibration included and exceeded those expected during routinary monitoring of batch cultures (0 – 240 $mg.L^{-1}$ and 0 – 75 $mg.L^{-1}$ for total mAb and NG-mAb respectively) and also the variability of mAb glycoforms. Off-line total mAb concentration range used for calibration was 0 – 380 $mg.L^{-1}$, off-line NG-mAb range was 0 – 98 $mg.L^{-1}$. Analysis of mAb glycoform relative abundance profiles revealed a significant difference between exponential and stationary-death phase of cultures (One way ANOVA, $p \leq 0.05$), particularly for NG-mAb, G0F, G1F, G2F and Man5 mAb glycoforms (data not shown). Moreover, enhancement of prediction capacity is expected since the use of feed-harvest cultures increased mAb glycoform variability during the calibration process.

### *3.3 Development and analysis of calibration models*

Firstly, in order to generate the calibration methods for mAb glycoforms, special attention was given to spectrum pre-processing according to Huang [22]. The presence of additive, multiplicative and wavelength-dependent effects due to scattering was evaluated within the calibration set spectra. The most common techniques to eliminate undesired spectral variations caused by light scattering (Multiplicative Scatter Correction-MSC, Probabilistic Quotient Normalization-PQN, Standard Normal Variate-SNV, Extended Multiplicative Scatter Correction-EMSC, derivatives) were evaluated. The standard deviation for each wavelength was used to elucidate the effect of scattering on calibration spectra; this data was considered for final spectral pre-treatment. The calibration set comprised 168 spectra collected from six bioreactor cultures. PLSR, LWR and SVR models and statistical analysis

were performed in MATLAB® (Statistics and Machine Learning Toolbox™, MATLAB R2016a, The MathWorks, Inc., Natick, Massachusetts, United States) using chemometric software (PLS_Toolbox® 8.2.1, Eigenvector Research, Inc., Manson, WA, United States). Model performance was evaluated for accuracy by Root Mean Square Error of cross-validation (RMSECV) and square correlation coefficients ($R^2$). A low value of RMSECV is related to enhanced accuracy, while a high value $R^2$ value indicates that the model properly handles spectrum variability to perform concentration estimation.

Firstly, PLSR models were performed using a venetian blinds cross-validation. Determination of latent variable (LV) number was based on the goodness of estimation (Q2Y): the minimum number of LVs was obtained when Q2Y ceased to improve. LWR was applied to fit global non-linear relationships by local linear regressions using PLSR and the classic cubic weight equation. Determination of local areas in term of local points, and LV number, was performed by optimization of these parameters with RMSECV as the response variable. For SVR models, an epsilon-support vector regression using a Gaussian radial basis function kernel was used. SVR models were optimized using a random subset cross-validation approach with maximal error values corresponding to deviations up to 10 % from actual values.

### *3.4 In situ monitoring of mAb glycoforms*

The focus of the work was primarily to analyse predicted or estimated kinetic profiles using batch culture. This provides a frame containing different physiological cell states within lag, exponential, stationary, and death phases of batch culture which dynamically impact the nature of mAb glycoform profiles. This strategy may be useful to infer model performance in different matrix compositions, which may help in future work.

Once calibration models were optimized, they were used independently to perform *in situ* monitoring of mAb glycosylation during a CHO cell culture. Characterization of mAb was

carried out in terms of macro-heterogeneity (total mAb concentration, glycosylated mAb and non-glycosylated mAb) and micro-heterogeneity (high mannose glycoforms and glycoforms containing any fucose, sialic acid and galactose moiety). A NIR analyser was programmed for performing automatic *in situ* scanning of culture medium every 20 min. Batch culture monitoring produced 500 spectra from which only 27 were used for calibration. Thus approximately 95 % NIR data was not used to establish the models and may further depict the prediction performances of models. For the evaluation of monitoring, models mimick real time monitoring of the mAb producing cell culture process. Spectra captured every 20 min were then used as inputs. Then calibration models returned mAb concentration values that were used to real time generate the kinetic profiles of mAb macro- and micro-heterogeneity (Figures 3-5). As global therapeutic effects of mAb is mainly a function of the micro-heterogeneity profile of the lot, real-time glycosylation data from best models were used to real-time monitor the global glycosylation profile of the produced lot. Firstly, the macro-heterogeneity profile was determined as the relationship of NG-mAb concentration estimated by the SVR model, with total mAb concentration estimated by the LWR model. Secondly, the micro-heterogeneity profile was determined as the relationship of fucosylated, galactosylated, sialylated and high mannose glycoforms with glycosylated mAb concentration, using SVR models.

## 4. Results and discussion

### *4.1 Development and analysis of NIR models based on PLSR, LWR and SVR methods*

Spectra for calibration were evaluated for scattering effects (Figure 2-A). The most common techniques to eliminate undesired spectral variations caused by light scattering (MSC, SNV, EMSC, derivatives) were evaluated. General analysis of spectra revealed some scattering effects such as additive effect (baseline shift), multiplicative effect (offset of spectra) and a likely wavelength-dependent effect from approximately 1000 to 1500 nm (Figure 2-B). These

351 effects were particularly observed in spectra of stationary and cell death phases where
352 maximum mAb concentration was achieved (data not shown). Only EMSC was effective for
353 limiting the likely wavelength-dependent effect, also the use of derivatives with any
354 normalization pre-processing, particularly 2$^{nd}$ order derivative with MSC (Figure 2-B). This
355 analysis was firstly used for selecting spectra pre-treatments for models. After spectral
356 analysis, random trials of promising pre-treatments and their combinations were assed for
357 reducing RMSECV of models.
358 In general terms EMSC leaded to PLSR models with lower RMSECV, likely due to a proper
359 compromise between reduction of spectra variability and scattering effects, particularly
360 multiplicative and wavelength-dependent effects. The use of derivatives after normalization,
361 limited the predictive power of PLSR models (data not shown), likely due to a strong
362 reduction on spectra variability (Figure 2-B) and thus reduction on chemical information. On
363 the other hand, LWR and SVR showed greater management of scattering effects, particularly
364 wavelength-dependent effect since only MSC was sufficient for reduction of RMSECV values
365 in almost all models. Final spectral pre-treatments used for calibration are shown in Table 1.
366 Construction of mAb glycosylation models was performed using different regression methods
367 (PLSR, LWR and SVR) as reported in Materials and Methods. Performances of models
368 during calibration are summarized in Table 2. PLSR models leaded to poor estimation
369 capability, even for total mAb concentration. PLSR is a variable space-based regression
370 method which calculates the relationship between each of the variables (absorptions at
371 different wavelengths) and compound concentrations. Such a relationship should be relatively
372 constant during the whole culture process in order to maintain accurate estimations, including
373 the scattering nature of the matrix. Perhaps the most evident deviation from this assumption is
374 the fact that scattering effects occurred and impacted spectra in different ways, depending on
375 the increase in scattering compounds (cells, cell debris, among others) according to batch

culture progression. A plausible reason for poor performance is the limited capacity of PLSR for handling multiplicative and wavelength-dependent effects [34], likely caused by scattering compounds. Handling spectra with such different scattering natures with the same spectral pre-proccesing as commonly done in PLSR, would not only lead to correcting response in a narrow frame but also masking chemical information in the uncorrected frame.

As a result, LWR and SVR were, for the first time, evaluated for cell culture monitoring. LWR and SVR are sample space-based regression methods which firstly focus on affinity and dissimilarity between samples (batch progression information), and secondly on the relationship between variables (absorptions at different wavelengths) and compound concentrations. LWR uses only similar samples in the PLS space to perform local regression using weighted PLSR, while SVR consists of a number of support vectors corresponding to samples from the calibration set and non-linear model coefficients defining the relationships between spectra and compound concentrations. As LWR and SVR use only similar samples with a similar matrix nature, including similar scattering effects, it is likely that non-desirable effects of spectral pre-processing are limited.

Results showed that, in contrast to PLSR performance, SVR and LWR were superior for estimating the concentration of all glycoforms (Table 2). This can be explained by the fact that SVR and LWR not only consider the relationship between spectra and compound concentrations, but also cell culture progression in terms of cell density, viability and metabolite concentrations. With the exception of total mAb which was better estimated by LWR, SVR was likely the best option for all mAb glycoforms, particularly glycosylated mAb.

*4.2 Real time monitoring of mAb glycosylation*

Firstly, mAb glycosylation macro-heterogeneity monitoring was addressed as shown in Figure 3. As expected, evaluation of PLSR models revealed a limited capacity for monitoring mAb macro-glycoforms, particularly non-glycosylated mAb, due to non-linear relationships between spectra and non-glycosylated mAb concentration (data not shown). In fact, non-linear relationships are likely the result of physical (scattering, mass and heat dynamics) and chemical (chemical composition changes) phenomena that strongly change the interaction of NIR radiation with mAb during progression of batch cell cultures. LWR breaks global nonlinearity by performing several local regressions using only similar samples. In this context, LWR was successful in monitoring total and glycosylated mAb concentration (Figure 3) that display some non-linearity mainly associated to physical phenomena. However, for non-glycosylated mAb, only trends were observed. Limited capacity to estimate NG-mAb concentration by the LWR model is explained by the fact that an inherent nonlinear relationship between spectra and concentration existed (data not shown), which cannot be properly modelled by the local linear regressions. As shown in Figure 3, the novel use of LWR and SVR as enhanced regression methods, allowed the proper monitoring of total mAb and NG-mAb respectively. These results demonstrated the capability to monitor mAb glycosylation macro-heterogeneity in real-time, using *in situ* NIR spectroscopy. As more accurate and stable estimations of glycosylated mAb concentration were achieved using SVR, calibration for glycosylated glycoforms was addressed using the SVR approach.

As for mAb micro-heterogeneity, among a total of 25 potential glycosylated mAb glycoforms reported for mAb produced in CHO cell cultures [2], only some glycoforms were detected off-line (data not shown). Thus, mAb micro-heterogeneity models were generated based on the detected glycoforms that contained particular sugar moieties conferring clinical properties. Detected glycoforms were classified into 4 groups for mAb micro-heterogeneity model development, corresponding to high mannose, fucosylated, sialylated and galactosylated

425 isoforms (Table 3). As a glycosylated chain may contain different sugar moieties, it is
426 possible that one particular glycoform be considered for two or more calibration models. SVR
427 was capable of properly extracting mAb glycosylation information from NIR spectra, which
428 allowed mAb micro-heterogeneity monitoring as shown in Figure 4. Even sialylated and high
429 mannose glycoforms whose concentrations were low (<15 $mg.L^{-1}$), were specifically detected.
430 These results demonstrated the capability of *in situ* NIR spectroscopy to quantitatively
431 monitor mAb micro-heterogeneity.
432 According to the QbD initiative, real time monitoring should finally be used for performing
433 advanced retro-control. Therefore, concentration values of mAb glycoforms must also be
434 monitored using a reference frame as for setting target values for glycoforms ratios, which are
435 related to mAb clinical effects. Monitoring of a process under this approach is a more
436 challenging task since calculation of ratios could increase the bounce of glycoforms ratio
437 profiles. For example, monitoring the extension of mAb macro-heterogeneity or the ratio of
438 glycosylated mAb with total mAb concentration, would add the error of predictions of both
439 glycosylated mAb model and total mAb model. This fact could compromise the resolution of
440 the final mAb glycosylation profiles and so further control strategies. Therefore, the capability
441 of models to clearly show these final profiles was investigated in the form of a control chart as
442 shown in Figure 5.
443 Only best models were used for calculating the mAb macro- and micro-heterogeneity profiles.
444 For the in-line mAb macro-heterogeneity profile, the fraction of glycosylated mAb was
445 calculated as the ratio of glycosylated mAb (estimated by SVR) with total mAb concentration
446 (estimated by LWR). Only SVR models were used for mAb micro-heterogeneity monitoring.
447 For the in-line mAb micro-heterogeneity profiles, the fraction of either fucosylated,
448 galactosylated, sialylated and high mannose glycoforms was calculated as the ratio of
449 particular glycoform with glycosylated mAb concentration. Then micro-heterogeneity profiles

could be monitored in real time as the fraction of glycoforms containing particular sugar moieties within the glycosylated chain, which are closely related to clinical properties of mAb medicine.

Once mAb concentration was higher than 30 $mgL^{-1}$, models allowed proper monitoring of mAb glycosylation profiles. This approach was encouraging for monitoring mAb macro-heterogeneity since accurate tendencies were observed during the whole culture, particularly for the abrupt decrease of mAb glycosylation around 100 h after the beginning of the process. As for mAb micro-heterogeneity, particularly for the fucosylated glycoform profile, a limited capacity was observed between 45 h to 70 h of the culture. This behaviour was also observed, though to a lesser extention, for the galactosylated glycoform fraction profile. On the other hand, sialylated and high mannose isoforms profiles were properly estimated even at low concentrations. Results demonstrated the potential of SVR, LWR and NIR spectroscopy for real time monitoring of mAb glycosylation properties during CHO cell culture processes. Moreover, accuracy on concentration monitoring also permitted monitoring of accurate trends of mAb glycoforms ratios, closely related to mAb clinical effects. Then such mAb glycoforms ratios could be used as target values for later control.

Overall, these are encouraging results for the use of NIR spectroscopy for developing new retro-control systems. However, caution must be taken when discussing eventual prediction capability of LWR and SVR in particular for mAb production processes. The same consideration should be shown in the case of NIR spectroscopy, as it contains both physical and chemical information linked to chemical and physical phenomena within processes. There is always the possibility of new variables in new production processes that have not been considered yet are critical to the performance of this newly developed prediction platform.

## 5. Conclusions

Data demonstrating the feasibility of NIRS to monitor mAb glycosylation *in situ* has been presented. In this study, the monitoring of both macro- and micro-heterogeneity of glycosylated mAb was improved by the novel use of sample space-based regression methods, particularly SVR, that could handle non-linear relationships between glycoforms and spectra. As far as it can be asserted, this is the first report of real-time and *in situ* monitoring of mAb macro- and micro-heterogeneity using NIR spectroscopy as well as the first report of LWR and SVR methods for cell culture monitoring. Such methods dealing not only with chemical but also some physical information contained within spectra, highlight the importance of considering the strongly dynamic nature of cell culture processes for accurate monitoring by calibration models.

There is an increasing number of new mAb producing processes including mAb biosimilars and biobetters [35], and one can assume that *in situ* spectroscopy methods will be implemented systematically to fulfil the demand in regard to quality. This study lays the foundation for future studies to expand the capabilities of *in situ* spectroscopy and multivariate analysis to monitor mAb properties so that enhanced retro-control strategies can be established, leading to a more efficient design and control of processes using PAT and the Quality by Design principles.

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## Conflict of interests

The authors declare no financial or commercial conflict of interest.

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606

607 **Tables**
608

609 Table 1. Spectral pre-treatment used for models

| Compound | Regression method | Pre-treatment |
|---|---|---|
| Total mAb | PLSR | EMSC + SNV |
| | LWR | SNV |
| | SVR | EMSC |
| NG-mAb | PLSR | EMSC |
| | LWR | Detrend + MSC |
| | SVR | MSC |
| Glycosylated-mAb | PLSR | EMSC |
| | LWR | EMSC |
| | SVR | MSC |
| F-glycoforms | SVR | MSC |
| G-glycoforms | SVR | MSC |
| S-glycoforms | SVR | MSC |
| HM-glycoforms | SVR | MSC |
| NG-mAb: Non-glycosylated mAb; G-mAb: Glycosylated mAb; F-glycoforms: Glycoforms containing Fucose; G-glycoforms: Glycoforms containing galactose; S-glycoforms: Glycoforms containing sialic acid; HM-glycoforms: Glycoforms of high mannose structures.<br>EMSC: Extended Multiple Scatter Correction, SNV: Standard Normal Variate, MSC: Multiple Scatter Correction<br>Auto scale was always applied as last pre-treatment step | | |

610

611

## Table

*Table 1: Data sources*

| Data type | Data source | Resolution | | Description |
|---|---|---|---|---|
| | | Spatial | Temporal | |
| **Model Inputs** | | | | |
| DEM | Global Digital Elevation Model V2 (NASA, 2011) | ~ 90 m | Static based on 2011 | 20 m resolution grid derived from contour line map 1:10,000 |
| Land cover | Corine Land Cover 2012 (CLC, 2012) | 100 m | Static based on 2012 | Land use map (Büttner and Kosztra, 2014) |
| Soil | Harmonized world soil database (FAO et al., 2012) | ~1 km | Static based on 2012 | a 30 arc-second raster database with over 16 000 different soil-mapping units combining existing regional and national updates of soil information as the European Soil Database (ESB) and FAO-UNESCO Digital Soil Map of the World |
| Climate (Observed) | SAFRAN – Météo France (Quintana-Seguí et al., 2008) (https://donneespubliques.meteofrance.fr/) | ~12km | Daily (2000 -2010) | Daily temperature (min., max.), solar radiation, humidity, wind speed of EMBRAPA CPAC station |
| City effluents | UWWTP – EUDB (https://ec.europa.eu/) (91/271/EEC) | Vectorial points | Annual (2010) | Annual outfall volume of wastewater water treatment plants and the amount of pollution produced by the agglomeration |
| Crop management | AGRESTE (http://agreste.agriculture.gouv.fr/ ; the projects Life Concert'Eau (2006-2009) and Aguaflash (Jarry, 2009) | Regional | Annual (2017) | Fertilizer application rates and timing, planting and harvesting information |
| Irrigation | CACG (https://www.cacg.fr/fr/) | Regional | Daily (2016) | Amount of water applied for irrigated crop |
| Dam management | EDF (REGARD-RTRA/STAE) | Vectorial points | Annual (1923 - 2017 ) | Dam management of eleven reservoirs with a reservoir volume ranging between 0.3 $km^2$ to 10.2 $km^2$ |
| **Calibration and Validation data** | | | | |
| River discharge | Banque Hydro (http://www.hydro.eaufrance.fr/) | Vectorial points | Daily (1921 -2010) | 20 flow gauging stations were selected in this study according to pedo-climatic regions (Probst, 1985) |
| Nitrate | France : Agence de l'eau (http://adour-garonne.eaufrance.fr/ ; Aminot and Kérouel, 2004) | Vectorial points | Ponctual (1990 – 2010) | Stream water $NO_3^-$- N load at 15 sampling sites |
| Crop yield | France: National Statistics (SAA, 2017) | Regional | Annual (2017) | Annual crop production in tons per year given by national statistics |
| Nitrate net balance | Teissier et al., 2008 | Vectorial points | Ponctual (low flow period - July 1999) | 96 samples in three reachs characterized by a "hyporheic zone" located on the Garonne river downstream of Toulouse ($mgN.m^{-2}.h^{-1}$) |
| | STREAMES European project (Martí et al., 2004; Sánchez-Pérez et al., 2009) | Vectorial points | Ponctual (2001 - 2003) | 24 measurements in two reaches (at the Lezat and Montegut sites) on every season with different hydrological conditions ($mgN.m^{-2}.min^{-1}$) |

*Table 2: Comparison between national yield (SAA, 2017) and simulated yield and PBIAS performance during the calibration period (from 2000 to 2010)*

| Crops | National Yield ($t.ha^{-1}$) | Simulated Yield ($t.ha^{-1}$) | PBIAS (%) |
|---|---|---|---|
| Almonds | 1.0 | 1.2 | -20% |
| Fruit Trees | 12.0 | 16.5 | -38% |
| Maize | 9.0 | 11.0 | -22% |
| Rice fields | 5.0 | 3.4 | 32% |
| Vineyard | 7.0 | 7.6 | -9% |
| Winter wheat | 5.0 | 4.2 | 16% |

*Table 3: SWAT model performance evaluation statistics for the daily streamflow at twenty gauging stations (cf. Figure 1 for station localization) during calibration (2000 - 2010) and validation period (1990 - 1999). Bold values indicate the best performances.*

| No | Station | Streamflow calibration (2000 - 2010) | | | Streamflow validation (1990 - 1999) | | |
|---|---|---|---|---|---|---|---|
| | | NSE | $R^2$ | PBIAS | NSE | $R^2$ | PBIAS |
| 1 | Ariège at Foix | 0.57 | 0.60 | -15.61 | 0.45 | 0.62 | -11.3 |
| 2 | Garonne at Saint-Béat | 0.45 | 0.19 | 4.42 | 0.45 | 0.13 | 8.8 |
| 3 | Garonne at Valentine | 0.65 | 0.66 | 5.43 | 0.42 | 0.67 | 5.9 |
| 4 | Salat at Roquefort | 0.32 | 0.39 | -5.27 | 0.37 | 0.42 | 13.6 |
| 5 | Ariège at Auterive | 0.61 | 0.67 | -4.93 | **0.71** | 0.72 | -5.6 |
| 6 | Garonne at Portet | **0.75** | 0.75 | -3.34 | 0.79 | 0.77 | -5.1 |
| 7 | Save at Larra | 0.39 | 0.42 | -13.32 | 0.49 | 0.55 | -16.3 |
| 8 | Garonne at Verdun | 0.49 | 0.69 | -4.69 | 0.41 | 0.71 | -5.2 |
| 9 | Tarn at Villemure | 0.45 | 0.64 | -2.88 | 0.44 | 0.59 | -3.6 |
| 10 | Tarn at Millau | **0.85** | 0.74 | -2.26 | 0.77 | 0.68 | -7.4 |
| 11 | Truyères at Sarrans | 0.46 | 0.50 | -2.94 | **0.50** | 0.44 | -2.5 |
| 12 | Lot at Entraygues-sur-Truyère | 0.41 | 0.64 | -2.96 | 0.31 | 0.59 | -5.2 |
| 13 | Truyère at Entraygues-sur-Truyère | 0.37 | 0.66 | -3.99 | 0.35 | 0.60 | -4.5 |
| 14 | Aveyron at Villefranche | 0.49 | 0.60 | -54.24 | **0.39** | 0.65 | -58.0 |
| 15 | Le Viaur at Laguepie | 0.38 | 0.64 | -10.94 | 0.3 | 0.68 | -9.6 |
| 16 | Tarn at Loubéjac | 0.38 | 0.63 | -8.67 | 0.36 | 0.67 | -8.5 |
| 17 | Lot at Cahors | 0.54 | 0.69 | 6.66 | 0.4 | 0.68 | 6.7 |
| 18 | Garonne at Lamigistère | **0.71** | 0.76 | -4.41 | 0.81 | 0.73 | -3.6 |
| 19 | Baiseat Nérac | 0.56 | 0.66 | -4.47 | **0.64** | 0.70 | -4.9 |
| 20 | Garonne at Tonneins | **0.87** | 0.78 | -5.58 | 0.86 | 0.76 | -4.7 |

*Table 4: SWAT model performance evaluation statistics of NSE, R2, and PBIAS (%) for the daily nitrate load at nitrate gauging stations (see Figure 1 for station localization) during calibration (2000 - 2010) and validation periods (1990 - 1999). Bold values indicate the best performances.*

| No | Station | Nitrate load calibration (2000 - 2010) | | | Nitrate load validation (1990 - 1999) | | |
|---|---|---|---|---|---|---|---|
| | | NSE | $R^2$ | PBIAS | NSE | $R^2$ | PBIAS |
| 1 | Ariège at Foix | 0.17 | 0.68 | 29.29 | 0.23 | 0.74 | 27.40 |
| 2 | Garonne at Saint-Béat | 0.41 | 0.57 | 7.15 | 0.33 | 0.57 | 8.74 |
| 3 | Garonne at Valentine | 0.31 | 0.67 | -9.90 | 0.28 | 0.63 | 11.20 |
| 4 | Salat at Roquefort | 0.43 | 0.51 | -26.1 | 0.25 | 0.51 | -15.77 |
| 6 | Garonne at Portet | 0.49 | 0.63 | -22.12 | 0.32 | 0.59 | 12.72 |
| 7 | Save at Larra | 0.05 | 0.57 | 40.82 | 0.21 | 0.45 | 40.65 |
| 8 | Garonne at Verdun | 0.31 | 0.86 | 36.41 | 0.12 | 0.8 | 40.87 |
| 10 | Tarn at Millau | 0.59 | 0.83 | 46.3 | **0.67** | 0.78 | 46.27 |
| 13 | Truyère at Entraygues-sur-Truyère | 0.51 | 0.65 | -39.64 | 0.34 | 0.65 | -26.15 |
| 14 | Aveyron at Villefranche | 0.28 | 0.49 | 14.9 | 0.59 | 0.57 | 19.38 |
| 16 | Tarn at Loubéjac | **0.64** | 0.74 | 9.99 | 0.47 | 0.76 | 20.67 |
| 17 | Lot at Cahors | 0.24 | 0.73 | **-9.77** | 0.32 | 0.67 | 13.73 |
| 18 | Garonne at Lamigistère | 0.53 | 0.64 | 9.69 | 0.46 | 0.61 | 39.33 |
| 19 | Baise at Nérac | 0.37 | 0.81 | -42.66 | 0.31 | 0.66 | -22.28 |
| 20 | Garonne at Tonneins | 0.59 | **0.73** | 9.89 | 0.72 | **0.85** | **9.88** |

*Table 5: Nitrate Net Balance summary ($gN.m^{-2}.d^{-1}$) of the upper part and floodplain area of Garonne watershed according to in-situ measurements (coming from Martí et al., (2004) and Tessier et al. (2008) databases ) and SWAT simulations. (SD: standard deviation)*

| | Upper part [1] *(Strahler order 3)* | | Floodplain [2] *(Strahler order 7)* | |
|---|---|---|---|---|
| | **Observed** | **SWAT Simulated** | **Observed** | **SWAT Simulated** |
| | unit: $gN.m^{-2}.d^{-1}$ | | unit: $gN.m^{-2}.d^{-1}$ | |
| **Mean** | -0.011 | -0.010 | -0.034 | -0.071 |
| Median | -0.002 | -0.003 | 0.094 | -0.023 |
| Maximum | 0.002 | 0.002 | 0.286 | 0.367 |
| Minimum | -0.115 | -0.107 | -0.703 | -0.593 |
| SD | 0.033 | 0.030 | 0.378 | 0.362 |

(1) Sánchez-Pérez et al., 2009 (n= 24)
(2) Tessier et al. (2008) (n= 96)

## *GRAPHICAL ABSTRACT:*