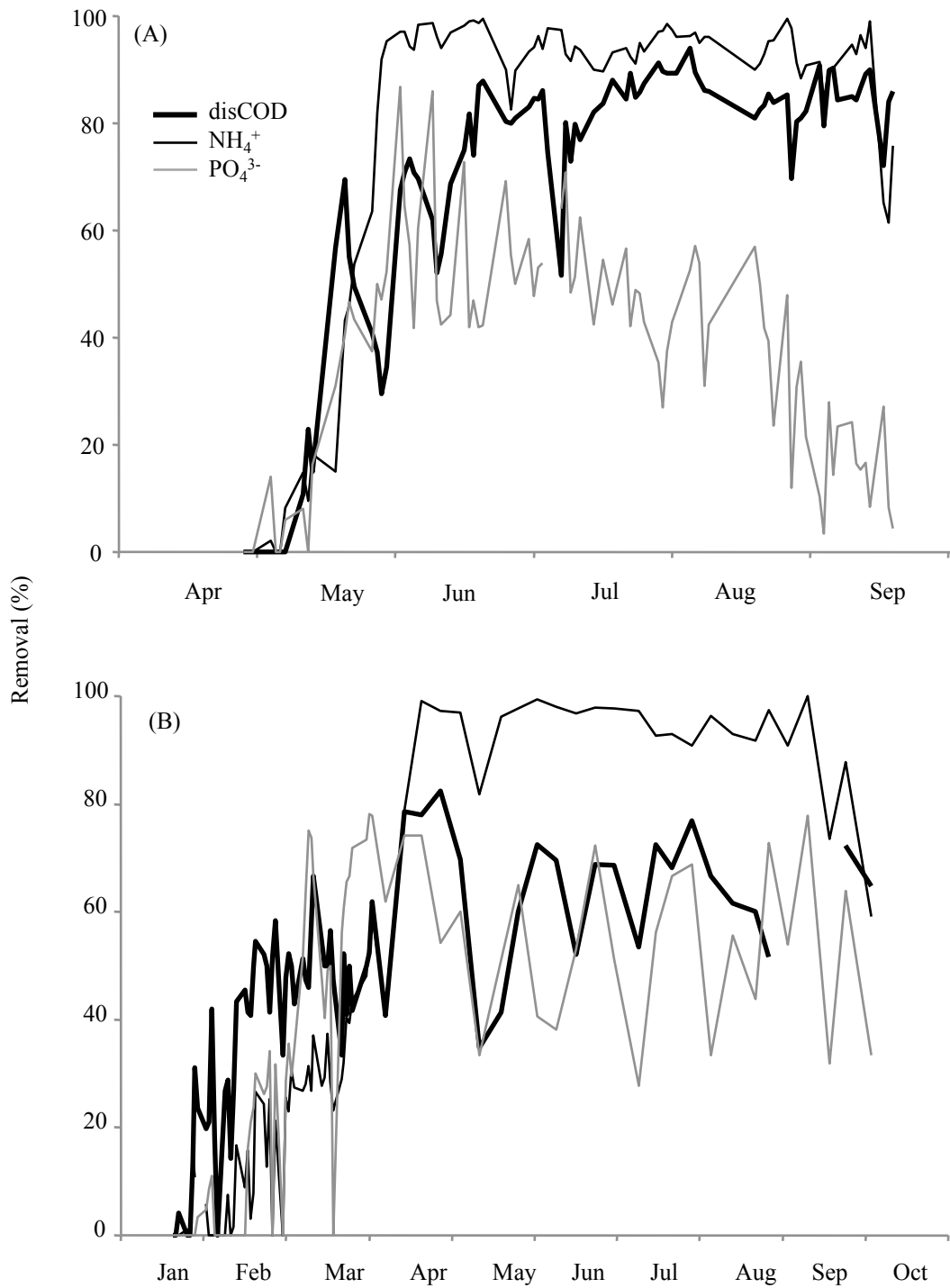


266 The comparison of the concentrations of NH_4^+ , PO_4^{3-} and dissolved COD in the
267 wastewater and in the HRAPs showed that the removal efficiency for nitrogen, phosphorus
268 and organic compounds was very variable. In the northern HRAP, 50% of PO_4^{3-} and more
269 than 80% of dissolved COD and NH_4^+ were removed about one month after start-up (Figure
270 1A). The PO_4^{3-} removal efficiency decreased when the water temperature dropped from 27°C
271 to 18°C and the daily incident light dose fell below 30 $\text{mE}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ at the end of August and
272 beginning of September (S1-Figure). In the southern HRAP, an average of 90% of NH_4^+ and
273 of 50% of dissolved COD and PO_4^{3-} were removed within three months (Figure 1B).
274 However, the removal efficiency of NH_4^+ started to decrease from the beginning of September
275 2016 (Figure 1B).



276

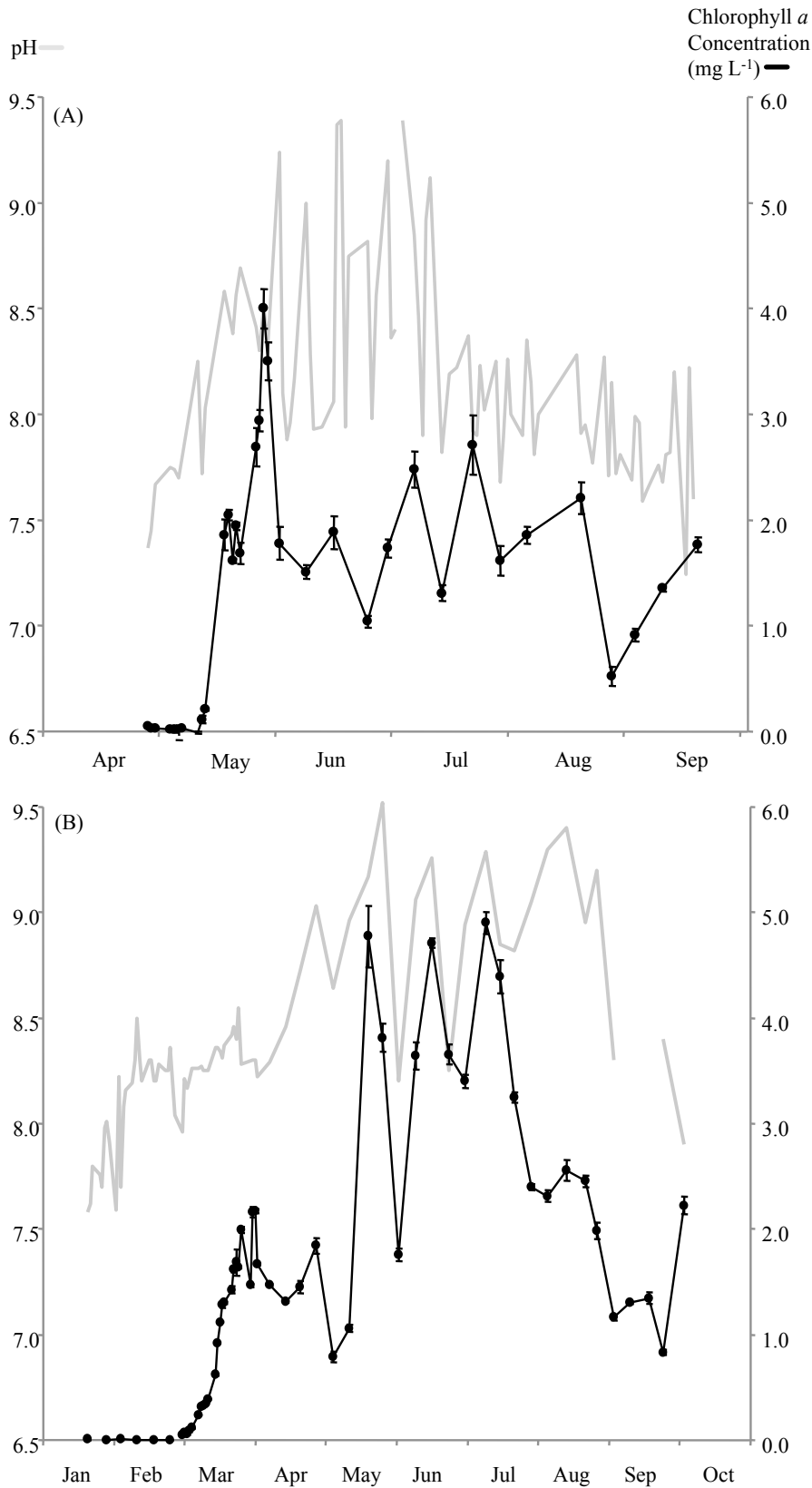
277 Figure 1: Removal (in %) of the dissolved COD (disCOD), PO_4^{3-} , and NH_4^+ in the northern
 278 (A) and southern (B) HRAPs. Removal was calculated as the difference between the
 279 concentrations in the wastewater and in the HRAP expressed as the % of the concentration in
 280 the wastewater.

281

282

283 For both HRAPs (Figure 2), at the end of the initial peak in pH (> 9), the chlorophyll *a*
284 concentration reached values equivalent or greater than 4 mg.L⁻¹ (end of May –beginning of
285 June).

286 As soon as the microalgal biomass started to grow in both HRAPs, the C, N and P
287 biomass dynamics were very similar and followed the Chlorophyll *a* concentrations (S2-
288 Table). During the period of microalgal growth, the C:N:P molar ratio of biomass averaged
289 100:12:1.



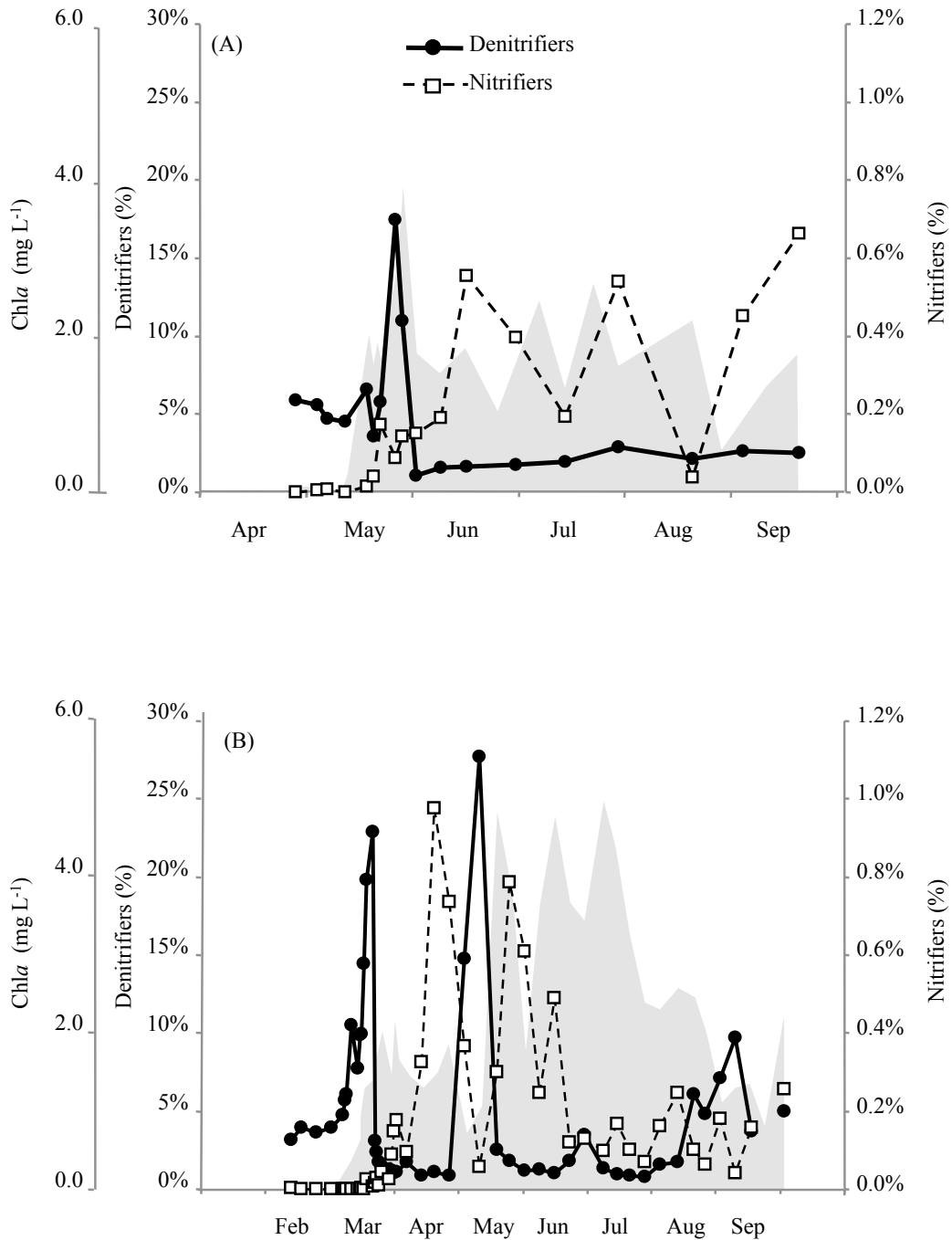
290

291 Figure 2: pH and Chlorophyll *a* concentration (mean and standard deviation from triplicate

292 samples) as a function of time in (A) the northern HRAP and (B) the southern HRAP.

293 3.3 Heterotrophic bacterial dynamics

294 In the northern HRAP, the bacterial abundance increased with a peak of 4×10^{12} cells.L⁻¹ on
295 June 26th 2015, two months after the beginning of the experiment (S3-Figure). In the southern
296 HRAP, the abundances were significantly lower (t-test, $p < 0.001$) and varied around 2.6×10^{11}
297 (S3-Figure). In the northern HRAP, the bacterial community was initially dominated by the
298 classes *Bacteroidia*, *Bacilli*, *Clostridia*, β - and γ -*Proteobacteria*. The proportion of
299 *Bacteroidia*, *Bacilli*, and *Clostridia* decreased rapidly within the first month, in favour of
300 *Sphingobacteriia*, and α -, β - and γ -*Proteobacteria* and *Cytophagia* by the end of the studied
301 period (S3-Figure). In the southern HRAP, the pattern was similar but with a much higher
302 proportion of β -*Proteobacteria* (ca. 40%) throughout the study period (S3-Figure).
303 Regarding the potential nitrifying bacteria such as *Nitrosomas*, they started to develop when
304 the algal biomass was growing, in May and March in the northern and the southern HRAP
305 respectively (Figure 3). Lower relative abundance of *Nitrosomas* was observed during
306 summer in the southern HRAP (Figure 3B). Numerous genera of bacteria may contain
307 heterotrophic denitrifying bacteria, although not all heterotrophic denitrifiers can perform
308 complete denitrification. When focussing on genera of potential denitrifying species observed
309 in activated sludge [32] and present in both HRAPs (*Acinetobacter*, *Achromobacter*,
310 *Alcalignes*, *Bacillus*, *Corynebacterium*, *Haliangium*, *Dechloromonas*, *Flavobacterium*,
311 *Rhizobium*, *Pseudomonas*, *Thauera*, *Thermomonas*), a peak of denitrifiers was observed when
312 the algal biomass started to grow in both HRAPs (Figure 3). Afterwards, their relative
313 abundance was decreasing and remained low (<3 %) except when the algal biomass suddenly
314 decreased (May, August and September in the southern HRAP).



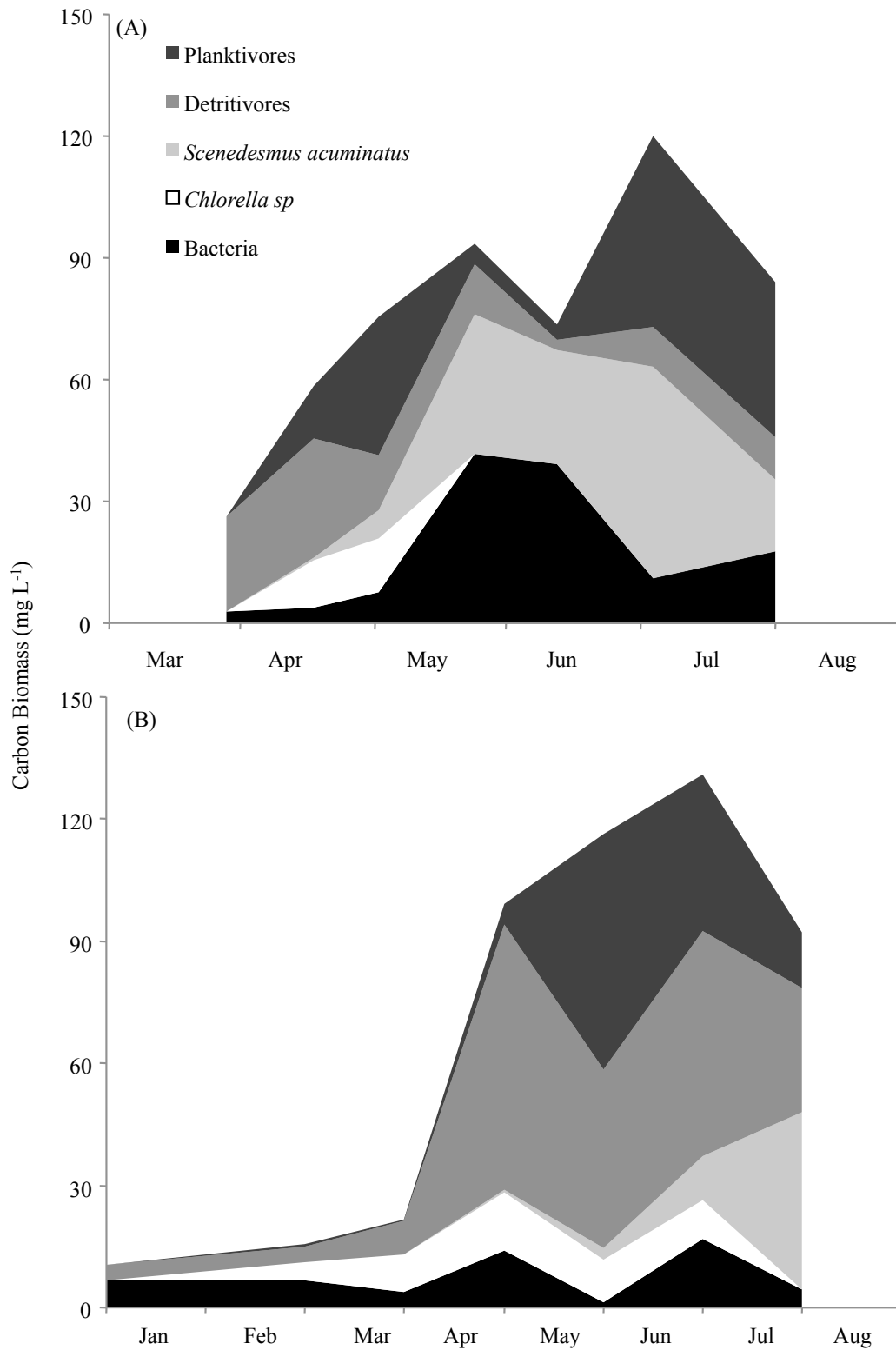
315

316 Figure 3: The relative abundance of bacterial OTUs (%) corresponding to potential nitrifying
 317 and denitrifying genera (see text) present in both HRAPs, as a function of time in (A) the
 318 northern HRAP and (B) the southern HRAP. Chlorophyll *a* concentrations were reported in
 319 shaded background.

320

321 3.4. Microalgal and cyanobacterial diversity

322 Of the 33 taxa identified by microscopy, 16 taxa were common to both HRAPs with the main
323 taxa being cyanobacteria or chlorophytes. It should be noted that more diatom taxa were
324 found in the southern HRAP (S4-Table). Some species, mainly some *Oscillatoriales*
325 (cyanobacteria), were only found in the wastewater in the southern HRAP and a green
326 microalgae (*Scenedesmus acuminatus*) was only found in the northern HRAP. During the
327 microalgal growth period, chlorophytes generally accounted for more than 70% of the total
328 abundance and biovolume in both HRAPs with a maximum biomass of 40-50 mg.L⁻¹ (Figure
329 4). During this initial period of microalgal growth, a succession of two chlorophyte species
330 (representing more than 80% of total chlorophytes) was observed in both HRAPs (S3-Table)
331 with the initial growth of *Chlorella* sp up to a maximum of 3.9x10⁹ cell.L⁻¹, followed by *S.*
332 *acuminatus* (max 1.7x10⁹ cell.L⁻¹).



333

334 Figure 4: The C biomass for bacteria, microalgae, protozoans and metazoans in the northern

335 and southern HRAPs during the study period. The abundances were converted into C biomass

336 using conversion factors taken from literature.

337

338 3.5. Growth of protozoans and metazoans

339 Rhizopoda (1 to 6×10^5 ind. L^{-1}) were the main group of large metazoans present in the
340 wastewater for both HRAPs before the microalgae grew (S5-Table). The succession of
341 different predator groups during the initial start-up period was quite similar in the two
342 HRAPs. After the initial rapid growth of Rhizopoda to a peak biomass of 30 - 65 $mg.L^{-1}$,
343 sessile and free-swimming ciliates (protozoans) then grew to a peak biomass greater than 40
344 $mg.L^{-1}$, followed by small (<100 μm) rotifers (S5-Table). These were followed by larger
345 (>100 μm) metazoans with similar abundances in the two HRAPs, with nematodes being the
346 most abundant and rotifers the next most abundant (S5-Table).

347

348 3.6. Dynamics of biomass compartments

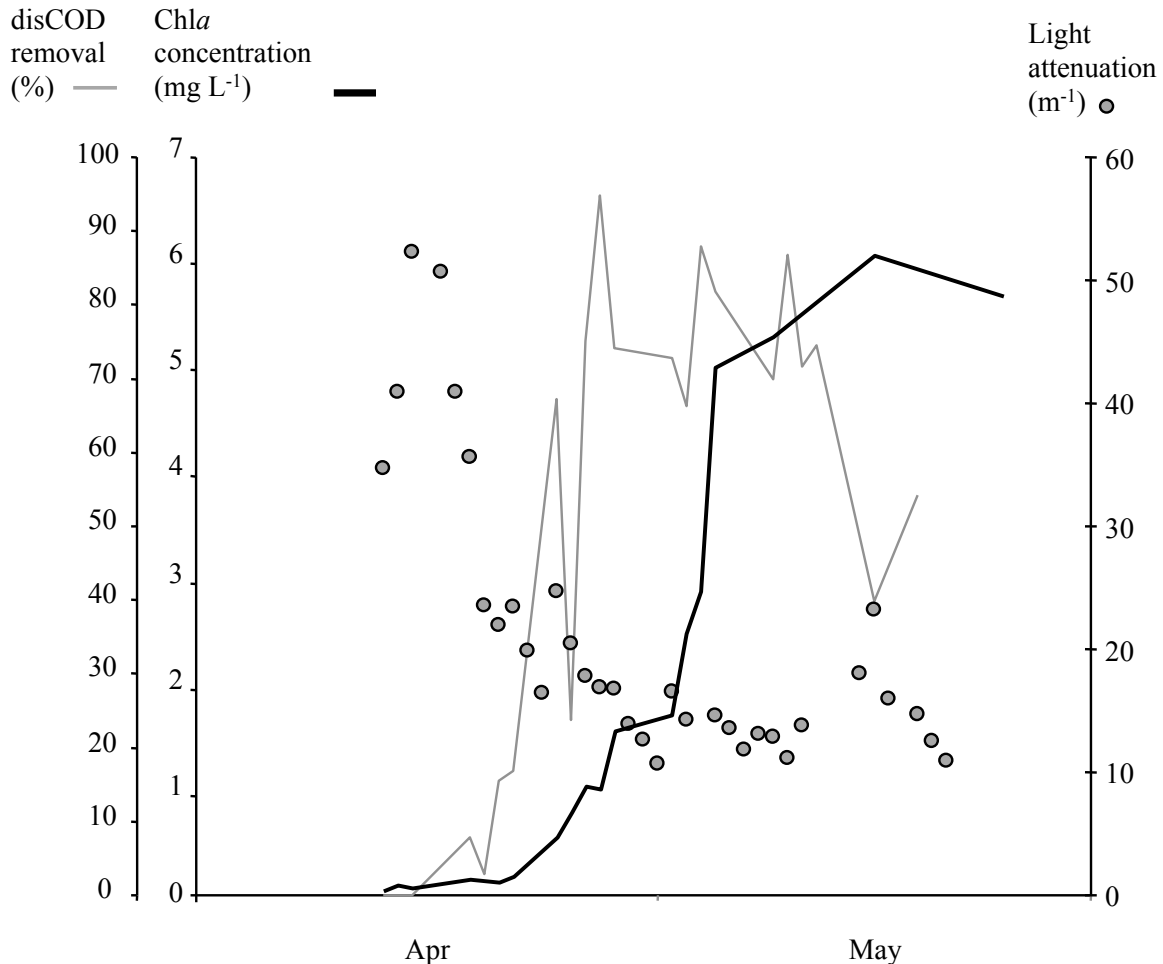
349 Detrivores (Rhizopods and Nematods) and bacteria dominated the total C biomass at the
350 beginning of the study period, averaging $56 \pm 23\%$ until the microalgae became dominant in
351 the two HRAPs (Figure 4). There was a clear microalgal succession in both HRAPs with the
352 rapid growth of first *Chlorella* sp and then *Scenedesmus acuminatus* with both contributing
353 up to 45 - 49% of the total C biomass. The microalgal growth was closely followed by the
354 growth of planktivores (i.e. ciliates and rotifers) that contributed up to about 50% of total C
355 biomass (Figure 4).

356

357 3.7. Turbidity clearance before microalgal growth

358 The second experimental run on the southern HRAP in spring 2017, when light attenuation
359 was recorded within the HRAP, showed a reduction of light attenuation simultaneously with a
360 strong increase of in dissolved COD removal during the first 15 days of the experiment
361 (Figure 5). The microalgal biomass started to grow when the vertical attenuation coefficient
362 for light fell below 30 m^{-1} (Figure 5). As the light attenuation decreased to 15 m^{-1} , the

363 dissolved COD removal reached an average of 75%. The peak chlorophyll *a* concentration (6
 364 mg.L⁻¹) was reached one month after the beginning of this new experimental run (Figure 5).
 365



366
 367 Figure 5: The light attenuation, dissolved COD (disCOD) removal and microalgal biomass
 368 (Chlorophyll *a* concentration) as a function of time in the southern HRAP in Spring 2017.
 369 disCOD removal was calculated as the difference between the disCOD in the wastewater and
 370 the disCOD in the HRAP expressed as the % of the disCOD in the wastewater.

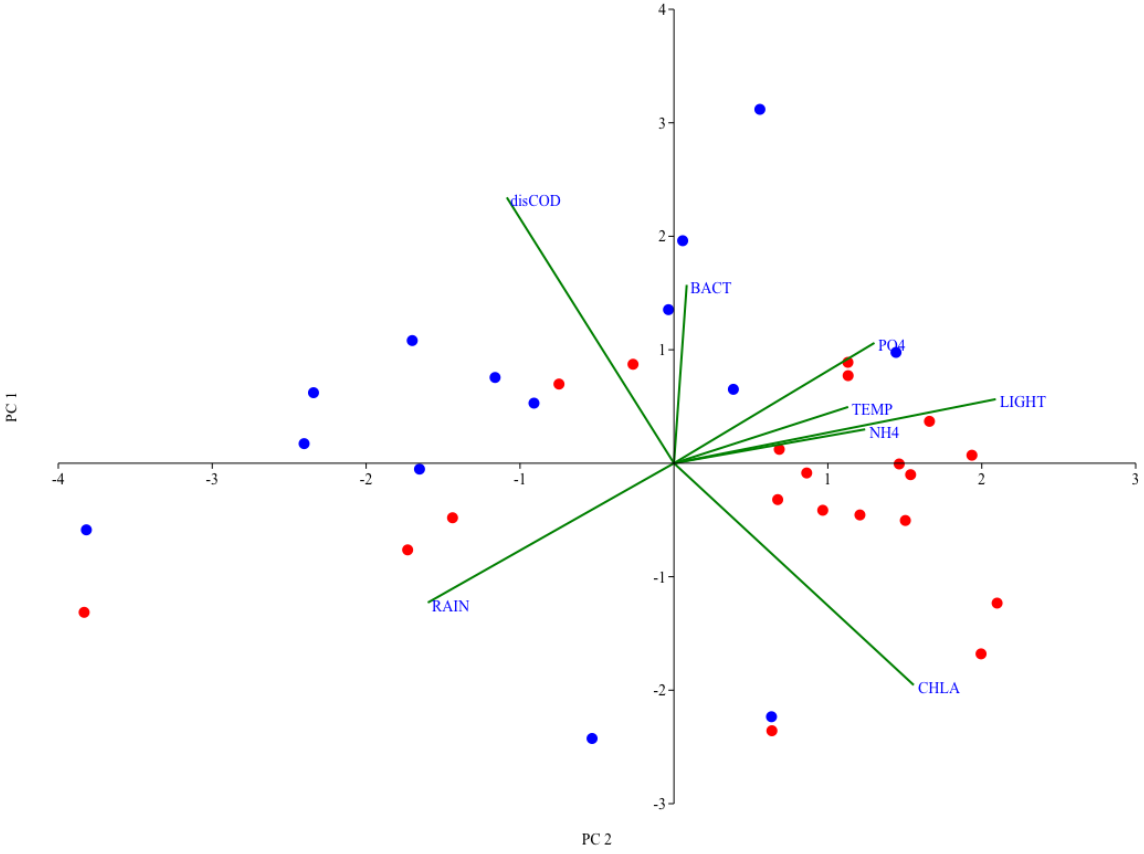
371
 372 3.8. Correlations between climate, microbial biomass and removal efficiency
 373 Significant differences (t test, $p < 0.05$, $n=35$) between the two geographical locations were
 374 reported for environmental and microbial data measured weekly during the period following
 375 the initial algal growth phase. The southern site was characterised by higher average of water

376 temperature, daily incident light dose and microalgal biomass (chlorophyll *a*) than southern
 377 site, characterised by higher average of bacterial biomass. No significant differences (t-test, *p*
 378 >0.05, n=35) between the two geographical locations were measured for rainfall, and
 379 disCOD, NH₄⁺ and PO₄³⁻ removal efficiencies. During this period, following the start-up
 380 phase, the first axis (31% of table inertia) opposed i) high values of NH₄⁺ and PO₄³⁻ removal
 381 efficiencies associated to the high values of water temperature, daily incident light dose and
 382 microalgae biomass, to ii) the high rainfall values (Figure 6). Interestingly, both the first axis
 383 and the second axis (21%) opposed i) the high values of bacteria biomass and disCOD
 384 removal efficiency, to ii) the high values of microalgal biomass (Figure 6).

385

386

387



388 Figure 6: A 35-sample 2D-PCA biplots of principal component 1 (31%) and 2 (21%) based
389 on correlation coefficients of environmental (temperature: TEMP, daily incident light dose:
390 LIGHT, rainfall: RAIN), microbial biomass (bacteria: BAC, microalgae: CHLA) and removal
391 efficiency (disCOD, PO_4^{3-} , NH_4^+) variables measured weekly in northern (blue dots) and
392 southern (red dots) sites during the period following the initial rapid algal growth.

393
394
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396

397 4. Discussion

398

399 4.1. Biological water clarification during the start-up phase

400 During the start-up period there was a dramatic reduction in the organic matter content
401 (removal of 80% of dissolved COD: disCOD) on both study sites. This rapid reduction is
402 likely due to the presence of a well-adapted bacterial and protozoan (detritivores) community
403 already present in the wastewater (the bacteria classes *Bacteroidia*, *Bacilli* and *Clostridia*,
404 amoebozoa and nematodes) which broke down the organic matter rapidly. The removal of this
405 organic matter led to an increase in light availability within the HRAP, which benefits fast-
406 growing autotrophic organisms such as *Chlorella* sp. The replacement of *Chlorella* sp. by
407 *Scenedesmus acuminatus* was also observed in another study of using HRAP to treat
408 municipal wastewater at a similar latitude [9]. Such a succession may be explained by i) a
409 strong grazing pressure on *Chlorella* sp. by ciliates growing rapidly in spring and ii) *S.*
410 *acuminatus* having a greater resistance to grazing pressure (colony formation, presence of
411 spicules). During this initial period of microalgal growth, the bacterial community
412 composition shifted with a reduction in classes typical of wastewater, such as *Firmicutes* [33],
413 but with the increase of nitrifying bacteria such as *Nitrosomas* (Figure 3). This is probably
414 due to the dramatic change in the conditions such as high concentrations of oxygen and
415 elevated pH as a consequence of the rapid growth of microalgae in the raceway.

416 The NH_4^+ , PO_4^{3-} and disCOD (> 70%) removal maxima were reached in about a
417 month in the northern HRAP starting in April 2015 and within three months in the southern
418 HRAP starting in January 2016. This difference is due to the difference in ambient
419 temperature at start-up, in spring at the northern site but in winter at the southern site. This is
420 supported by the rapid dissolved COD removal (within one month) observed at the southern
421 site during the second experimental run starting in April 2017. Despite some differences

422 observed in the chemical characteristics of the wastewater of the two HRAPs, different
423 latitudes and different climates, disCOD (200-360 mg.L⁻¹), phosphate (2-3 mg.L⁻¹), and
424 ammonia (40-60 mg.L⁻¹) were all removed efficiently within a month, during spring season.
425 Simultaneously, microalgae concentration increased to about 4-5 mg Chl_a.L⁻¹ in about two
426 weeks time in both locations in spring, corresponding to about one cell division every two
427 days. Very few studies have focussed on the start-up period of wastewater treatment in
428 HRAPs, and none of these have taken the frequent measurements required to measure growth
429 rates. Our results are close to those obtained under laboratory condition, showing that both
430 *Chlorella sorokiniana* and *Scenedesmus obliquus* could individually grow in 100% urban
431 wastewater, reaching a maximum biomass in 15 days (one cell division every 2-3 days) and
432 removing more than 70% of COD, NH₄⁺ and PO₄³⁻ [34]. We therefore suggest that in
433 temperate climates during the growing season, with the initial help of detritivores and bacteria
434 for a rapid water clarification, two weeks are required for microalgae to grow naturally in
435 wastewater without start-up inoculation, and remove most disCOD, NH₄⁺ and PO₄³⁻.

436

437 4.2. Influence of microbial N exhaustion on P removal

438 For both HRAPs, the PO₄³⁻ removal was on average 50% and highly variable throughout the
439 study periods, decreasing to less than 10% in September at the northern site. Given the molar
440 N:P ratio of the biomass (12±3) produced in both HRAPs and the molar NH₄⁺:PO₄³⁻ ratio of
441 the wastewater (greater than 30), phosphate should have been the main limiting factor for
442 microalgal growth. But the almost complete removal of NH₄⁺ and only partial removal of
443 PO₄³⁻ in both HRAPs, would suggest an overconsumption of NH₄⁺ compared to PO₄³⁻, making
444 N the main resource controlling the microalgal biomass production. The N biomass produced
445 in both HRAPs was much lower than the NH₄⁺ removed (S6-Figure). Therefore, a large
446 fraction of the NH₄⁺ consumption (>50%) was not converted into measurable N biomass,

447 suggesting high N losses. A significant release of nitrogen as dissolved organic compounds
448 by microalgae or bacteria was unlikely as no accumulation of dissolved organic nitrogen (the
449 difference between disN and inorganic N forms) was observed in the HRAPs (data not
450 shown). A large N loss through NH₃ stripping was also unlikely because the NH₃ contribution
451 to NH₄⁺ was between 5 and 30% according to the pH (between 8 and 9) and the very high
452 NH₄⁺ solubility in water makes NH₃ stripping difficult without intense aerosolization. The
453 biological release of N-compounds with a weaker solubility in water such as N₂ generally
454 occurs through bacteria activity under anoxic or hypoxic conditions [35]. The presence of first
455 NO₂⁻ and then NO₃⁻ production in both HRAPs (S6-Figure) are indicative of nitrification
456 processes through the oxidation of NH₄⁺ by nitrifying bacteria. This is supported by the
457 increasing relative abundance of nitrifying species such as *Nitrosomas* when microalgae
458 started to grow in both HRAPs. The stimulation of nitrification activity due to increased
459 oxygen-derived photosynthesis has been reported [36, 37], although some studies have
460 reported potential nitrification inhibition in the presence of microalgae [38]. The succession
461 of highly oxygenated conditions during daylight and the anoxic or hypoxic conditions during
462 the night, together with the continuous supply of high NH₄⁺ seems to favour the oxidation of
463 NH₄⁺ into N₂. Occasional peaks of potential denitrifying species were reported in both
464 HRAPs, supporting that denitrification might have occurred during the algal growth period. In
465 contrast, the maximum values of the phosphorus content of the biomass produced in the
466 HRAPs were close to the PO₄³⁻ concentrations removed (S6-Figure). This suggests that PO₄³⁻
467 was mainly consumed by microalgae in both HRAPs, but PO₄³⁻ microalgal consumption was
468 probably limited by the low NH₄⁺ availability due to the activity of nitrifiers/denitrifiers
469 converting NH₄⁺ as N₂. This is supported by the higher PO₄³⁻ removal rates observed at the
470 end of summer in southern HRAP as compared to the northern HRAP, when the relative
471 abundance of potential nitrifying species was very low.

472

473 4.3. Critical role of microbial interactions in the wastewater treatment efficiency

474 After the rapid development of microalgae biomass during the start-up phase, the highest
475 NH_4^+ and PO_4^{3-} removal efficiencies were related to the high activity of microalgae under
476 elevated water temperature and daily incident light dose, but low rainfall. This clearly
477 highlights the direct influence of environmental conditions on the N and P removal
478 efficiencies. Although the average of microalgae biomass was significantly higher in southern
479 site than in northern site because of warmer and higher light conditions, similar NH_4^+ and
480 PO_4^{3-} removal efficiencies were measured in both sites. However, very low PO_4^{3-} removal
481 efficiencies were reported in northern site at the end of the study (Figure 1A). Such low PO_4^{3-}
482 removal was attributed to the microalgal growth conditions becoming less favourable, with
483 low daily incident light dose, together with a strong NH_4^+ competition with nitrifying
484 bacteria. In addition to low light availability, the slow-growing *Scenedesmus acuminatus* led
485 to the growth of large rotifers (*Monogononta* sp., > 100 μm), which are able to graze on and
486 control the population of *S. acuminatus*. Overall, the planktivores (both ciliates and rotifers)
487 contributed to 50% of the total C biomass produced in the HRAPs (Figure 4). Such high
488 contribution of planktivores is due to their accumulation in the raceways suggesting that the
489 6-day hydraulic retention time (HRT) was not short enough to wash them out or that they
490 avoided the weir at the outflow. Similarly, large rotifers and cladocerans were found in
491 HRAPs using paddlewheels for mixing, with an HRT of 4 days, during spring and summer [7,
492 39].

493 Interestingly, the highest disCOD removal efficiencies were measured when the
494 microalgae biomass was low (Figure 6). This suggests a strong contribution of heterotrophs
495 organisms (bacteria and detritivores) to the disCOD removal, in condition unfavourable to algal
496 accumulation (low light and/or high planktivores). This is supported by the general increase

497 of disCOD removal efficiency observed during the last period of the study in the northern
498 site, with values greater than 80% (Figure 1A), where bacterial abundance was the highest
499 and when microalgae were collapsing. We assumed that the ability of both bacteria and
500 microalgae to exhaust NH_4^+ rapidly as discussed earlier (4.2), would have a direct impact not
501 only on the algal PO_4^{3-} removal but also on the disCOD removal by bacteria that require N
502 source as well. Therefore, in addition to environmental conditions, microbial interactions
503 within the HRAPs would influence substantially the wastewater treatment and an efficient C
504 or P removal will be dependent on microorganisms (bacteria or microalgae) that outcompete
505 for NH_4^+ . Similar biological and environmental constraints seemed to be applied on both
506 HRAPs. This suggests that the removal efficiency would be highly reproducible under
507 different temperate latitudes.

508

509

510 5. Conclusion

511 This study, which was carried out under outdoor conditions using urban wastewater influents,
512 demonstrated that there is a similar biological succession and dissolved COD, NH_4^+ and PO_4^{3-}
513 removal efficiencies during the wastewater treatment using a High Rate Algal Pond (HRAP)
514 under both temperate oceanic and Mediterranean climates. The results suggest that water
515 clarification through the consumption of organic matter by detritivores, heterotrophic bacteria,
516 nematodes, amoebozoa initially present in the wastewater, is a prerequisite for a rapid growth
517 of microalgae. Together with the microalgae, bacteria participated substantially to the
518 complete exhaustion of ammonia. The resulting competition for NH_4^+ would drive the level of
519 removal efficiency of disCOD by bacteria and PO_4^{3-} by microalgae. The use of a modelling
520 approach focusing on trophic relationships (i.e. prey-predator, N competition) could help

521 reveal the key biological interactions acting on the overall process efficiency and propose
522 effective control and optimization strategies that could be tested using further experiments.

523

524 **Acknowledgements**

525 This study was supported by PHYCOVER project, which was funded by the French National
526 Agency for Research (ANR-14-CE04-0011). We should like to thank Patrick Raimbault
527 (UMR Institut Méditerranéen d'Océanologie, Marseille) for the analyses of the carbon and
528 nitrogen content of the biomass. We thank the two anonymous reviewers for their
529 constructive comments, which improved the manuscript.

530

531 **Declaration of contributions**

532 AG, VJ, BS, JPS and EF have worked in the conception and design of the study. AG, AB,
533 CC, EL, ELF, JP, CP and CR have contributed on the acquisition of data. MS contributed to
534 the statistical analysis of data. AG, VJ, ELF, CR, BS, JPS, EF have realized the interpretation
535 of data and drafted the article.

536

537 **Conflict of interest statement**

538 We declare that this manuscript has not any potential financial or other interests that could be
539 perceived to influence the outcomes of the research

540

541 **Declaration of authors**

542 All authors have approved the manuscript and agree with peer review process and its
543 submission to Algal Research

544

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